1989

Nutritional Influences on Aging in Aspergillus ornatus

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NUTRITIONAL INFLUENCES ON AGING IN *ASPERGILLUS ORNATUS*

A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

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by

Jaydeep Shantilal Shah

1989

APPROVAL SHEET

This thesis is submitted in partial fulfillment of the requirements for the degree of

Master of Arts

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Approved, July, 1989

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TABLE OF CONTENTS

DEDICATION

In loving memory of my mother, who passed away long before her time. I miss you mom.

ACKNOWLEDGMENTS

The work presented here would not have been accomplished successfully in the one year goal I had set for myself without the support and encouragment of several individuals whom I am indebted to for various reasons.

First and foremost, my thanks to my brother and father go beyond words. The sacrifices they have made in order that I may pursue my dreams can never be repaid.

Of the confidence expressed in me by Professor Coursen ("Coach") this year, I am proud. Though the road was quite rocky at first, I feel that we have built a long lasting friendship, both professionally and personally. I wish him all the best for the coming years.

A special debt of gratitude belongs to my laboratory "neighbors", Dr. Scott ("Joe-California"), Dr. Hoegerman ("Hoagie"), Jewel Thomas (whose name says it all), and Bill ("hot stuff") Saunders. Without the use of the equipment in their perspective labs, I would still be ripping the hair out of head. Moreover, their friendship and caring has made them like family to me.

Thanks for everything, you will always be in my thoughts.

I am also grateful to the numerous professors who have given me generous advice. I would like to especially thank Dr. Black, for coming to the rescue when I needed a committee member.

Finally, I am more than thankful for my friends who freely gave their time and good ears when I needed to blow my top. For Holly LaVoie, for many reasons, I will always be thankful. I am, and always will be, a devoted friend filled with warm affection.

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABSTRACT

The goal of this investigation was to determine the impact of nutritional restriction on aging cultures of Aspergillus ornatus. Four nutritional levels were studied: severly restricted cultures (group SR) were grown continuously on a medium containing initially 10g/L glucose; moderately restricted cultures (group MR) on a 20g/L glucose concentration medium; slightly restricted cultures (group SLR) on a 30g/L glucose concentration medium; and C groups, which served as control cultures, on a medium containing initially 40g/L glucose. Dietary restriction was effective in sustaining o-pyrocatechuic acid (OPCA) carboxylyase activity with increasing age in SR and MR groups. Beneficial influences on regrowth capability due to nutritional restriction were also noted in aged cultures. A significant positive correlation between depressed growth and sustained enzyme activity occured in old 336 hour cultures. Dietary restricted cultures (SR and MR) attained significantly lower total protein and DNA content than that of control groups. No quantitative or qualitative determination could be made of active nuclei in DAPI-stained cell preparations examined by fluorescent microscopy. The findings of this study show the profound anti-aging effects of dietary restriction and provide further insights as to the possible mechanisms of cellular aging.

NUTRITIONAL INFLUENCES ON AGING IN *ASPERGILLUS ORNATUS*

INTRODUCTION

The biology of the aging process is far from being a completely understood phenomenon. Though it is fundamental to all life forms as a gradual deterioration of structure and function, there has not been a detailed account or experimentation of the process to provide more than just a set of hypotheses. Furthermore, the aging process itself is not accepted as a separate entity by many scientists, who view aging as a part of the developmental process.

In order to justify the process of aging as a separate entity, it becomes necessary to define aging as well as establish criteria for age changes. It is emphasized that aging is a property of an organism and not of its environment [1]. It is this criterion that distinguishes aging from age-correlated changes which are due to factors outside the organism. Aging is often defined as those changes that: (1) take place in the postreproductive period of an organism, and (2) result in a decreased ability of the organism to adapt to its environment. The postreproductive aspect is noted to differentiate those earlier processes denoted positively as "growth". This definition allows for laboratory pursuits and is operationally similar to those stated by Comfort [2] and Strehler [3].

The aging process is not a universal characteristic of all organisms, but of individual species [1]. That is, different lines of evolution may decrease in their ability to adapt to the environment for entirely different immediate reasons. However, the possibility that there exists one or more dominant mechanisms of aging that may regulate the process in all organisms is viable. In the current era of molecular biology, this assumption has led to many studies of aging at the cellular level. Cellular mechanisms, such as protein synthesis [3], carbohydrate metabolism [4], and photosynthesis [5], have been extensively studied with respect to aging, since they serve as a measure of a cell's ability to function in response to a particular environment. As a result of these and similar studies, many theories as to the mechanism(s) of the aging process have been put forth.

August Weissman (1889) suggested a limited existance of organisms based on what he termed "programmed cell death." He viewed aging as just the continuation of growth, development, and morphogenesis, and suggested that there are genes that become activated after the process of maturation which limit the life span of a particular species. This is known as the Genetic Program Theory. In support of programmed death

is the work of Hayflick and Moorhead [6] who found that cultured human fibroblasts divide approximately fifty times and then die even though cultural conditions would seem to permit for indefinite divisions.

In agreement with the notion of a genetic basis of cellular aging, but against programmed cell death is the Error Catastrophe Theory proposed by Leslie Orgel [7]. He suggests that errors in DNA replication via mutations is the basis of aging. The accumulation of DNA which is potentially normal, yet abnormal or lethal when transcribed and translated, can be devastating to cell processes when the error is in the production of an enzyme involved in any of the facets of protein synthesis. However, there is little evidence in support of this otherwise attractive theory [8,9].

Another possible explanation of the aging process is Harman's Free Radical Theory [10]. It states that cells age and die due to a progressive loss of the free radical defense mechanisms in cells. A great deal of research has been focused on the harmful effects of reactive intermediates formed in the reduction of molecular oxygen, such as the superoxide radical O and HO . The radicals, due to their strong nucleophilic, character readily attack polyunsaturated fats and extract hydrogen from them, which in turn results in

cross-linking with lipids, proteins, and nucleic acids. Recent studies have been focused on determining whether levels of superoxide dismutase, a free radical trapping intermediate, decrease with aging. Unfortunately, results have been conflicting and the question still remains unanswered [11,12].

There are also numerous other theories at the molecular level that have been proposed to explain biological aging. Some of these include: the Redundant Message Theory; the Transcriptional Event Theory; the Cross Linkage Theory; and the Somatic Mutation Theory [1].

As enzyme activity is the single most important component of cellular function, this mechanism has been studied extensively for signs of deterioration with age. Early studies of the Gershons [13] showed the presence of a mixture of active and inactive molecules composing the isocitrate lyase enzyme fraction in aging nematodes. They discovered similar mixtures of active and inactive molecules of the aldolase enzyme fractions of both mouse muscle and liver tissues [14,15].

Measurement of the Krebs cycle enzymes in Mycobacterium tuberculosis showed a decrease in activity of all enzymes in the pathway except isocitrate lyase, which increased nearly five fold. The author suggests

that some regulatory mechanism is involved and that a general depression of the synthesis of all proteins has not occurred [16]. In Neurospora crassa, the ratio of active to inactive enzyme molecules of glutamic dehydrogenase indicate mistranslation of the protein and decreased synthesis of the enzyme [17].

Thus enzymes, as specialized proteins, are effected by age in at least two ways. Either actual synthesis of the enzyme is affected quantitatively, or there is a modification of any of the factors necessary for activity which renders the enzyme inactive, a qualitative change.

While constitutive enzymes are vital to living systems, the inducible enzymes are an exceptional index of the ability of an organism to respond to an environmental change, or in other words, adapt to a changing environment. Enzyme induction, which incorporates the phenomenon of protein synthesis and response to regulatory factors, is also affected by the aging process. Compiled studies by Adelman et al. [18] have shown that an organism loses its ability to produce these enzymes in response to an inducer as it ages. The age effect on activity of the inducible enzyme is seen either at the level of decreased protein synthesis or at the level of induction regulation.

The mechanisms for the decreased inducibility have yet to be fully understood. A decrease in the ability of the inducing substance to enter the cell through reduction of membrane permeability or loss in the ability to actively transport it has been suggested by Langheinrich and Ring [19]. Gershon and Gershon [13] have shown that if isozymes exist for a particular enzyme, and if these isozymes are inactive and accumulate over time, then although the inducer may readily enter the cell, the induced enzyme's ability to break down the inducer has been diminished. The feedback mechanism of gene expression at the operon level becomes faulty, continually producing both defective and active enzyme. This reduces the overall specific activity of the enzyme.

In accordance with the ideas of Orgel and the Gershons on erroneous replication and transcription as the basis of aging, Barrows [20] proposed the hypothesis that reduced protein synthesis may increase life span by retarding genetic information transfer during early life and reducing the use of the genetic code, and thereby minimizing genetic imperfections as they may occur during late life. He tested his hypothesis on Wistar rats by attempting to reduce protein synthesis by lowering the dietary protein level, thus limiting the

available amino acids. Barrows found that by feeding a diet which contained 12 percent protein increased the life span by 25 percent of 16-month-old female rats previously maintained on a commercial diet of 23.4 percent protein.

The revelation of dietary restriction increasing longevity is ancient in terms of scientific advancement. Since the original reports of McCay et al. in 1935 on the increased longevity in dietary restricted rats, many repititions of similarly designed experiments have confirmed this result [21]. And while there is no difficulty in increasing longevity by dietary restriction, few studies offer a firm explanation as to the mechanism(s) of prolonging life in this manner. Most studies conclude by stating that the fundamental mechanisms underlying the profound influence of dietary restriction on long lived strains of rodents still must be discovered.

Associated with increased life span via dietary restriction is growth retardation [21]. Dietary restriction started on either weaning or middle aged mice or rats has been shown to not only depress body weight, but also maximize longevity. This connection between dietary restriction, growth retardation, and increased longevity is seen in many other organisms.

Walford [22] has stated that decreased caloric intake without malnutrition may greatly influence the life span of many species from fish to man. Studies have shown that dietary restriction delayed the occurrence of biochemical and physiological changes during growth of rotifers and not only increased the duration of early life (the egg laying period) but also the total life span [23]. Delaying growth via dietary restriction in the fish Lebistes reticulata has been reported to increase its longevity [24]. Evidently, the restriction of caloric intake modulates critical metabolic mechanisms which in turn regulate the rate of aging, and ultimately, longevity.

However, to postulate theories on cellular aging based on the study of whole animals, which may result in the masking of some cell processes, can be somewhat misleading. If one wants to design a study to examine cellular aging, one needs to work with cell cultures. Attempting to culture mammalian tissue for in vitro studies tends to be time consuming as well as unreliable in success. The need for a simple and accessible source of cells has led to the use of fungi in cellular aging experiments.

Fungi have proven to be an excellent tool for investigating aging of eukaryotic cell systems. The

material is readily subcultured, providing a relatively simple source of cell populations, and "ages" relatively rapidly, within 7 to 14 days. The multiple spore spray inoculation technique of Yanagita and Kogane [25] insures the production of a homogeneous mycelial mat, composed of cells of similar physiological ages. In addition, a considerable amount of work has been done on the inducible enzymes of the tryptophan catabolic pathway in Neurospora crassa and Aspergillus niger [26,27].

Work accomplished in this laboratory has proven the existence of the inducible enzyme o-pyrocatechuic acid (OPCA) carboxylyase in the filamentous ascomycete Aspergillus ornatus Raper. Studies of Spiegelman and Coursen [28] have established that the specific activity of the induced enzyme decreases with age from six times the basal level to only twice the basal level when A. ornatus is grown on standard four percent glucose defined medium. This decrease in specific activity is thought to be attributed to an inactivation of OPCA carboxylyase rather than a decrease in the production of the enzyme [29]. Further studies show that the time of induction and concentration of the inducer also effect the specific activity of OPCA carboxylyase in aging

cultures of A. ornatus [30].

In order to initiate a study on the effect of nutrition upon longevity, it is necessary to examine the nutritional requirements of A. ornatus. The fungus mycelium is primarily composed of carbon and nitrogen. Almost all of the nitrogen supplied to fungi is in the organic form. In culture, organic nitrogen may be supplied as amino acids, peptides, or peptones. The problem associated with amino acids is their toxicity to fungi [31]. Toxins related to amino acids are very heterogeneous because of the many different amino acids from which they are synthesized. It is very probable that amino acids cause the early termination of the organism rather than being a source for increased longevity.

Approximately half of the dry weight of fungal cells consist of carbon, which gives an indication of the importance of carbon compounds in fungal metabolism. Fungi can utilize a wide variety of organic compounds as sources of carbon. Given a solution of various nutritional forms of carbon, almost all fungi will preferentially utilize D-glucose [31].

The criteria for measuring aging, as stated earlier, are met by beginning measurements only after mitosis has ceased or reached a constant turnover level,

as determined in filamentous fungus through the estimation of cell numbers by the quantity of DNA [32]. The ability of A. ornatus to adapt to its environment with increasing age can be determined by the production of OPCA carboxylyase at specified time intervals in the life span of the organism.

In association with enzyme induction as a measurement of aging, and ultimately longevity, it has been previously suggested that the ability of a fungus to promote regrowth is a valid measurement of the aging process [33]. Studies have shown that the older the mycelium, the lower the rate of regrowth when a portion is inoculated into fresh medium. Regrowth of the organism in a tube limits growth linearly and in one direction,and provides an accurate measure of the rate of growth [34]. It is recognized that this is not an accurate measure of viability, but it provides some idea as to the relative viability of the cultures at different ages.

A more accurate measure of viability is the determination of the actual number of functioning nuclei. The rate at which nuclei are lost gives an indication of the rate of aging. The use of the DNA fluorochrome 4'-6 diamidino-2-phenolindole (DAPI) allows for specific staining of active nuclei [35].

The study presented here investigates whether it is possible to delay the onset of senescence in A. ornatus by dietary restriction. Longevity is determines by the parameters of OPCA carboxylyase specific activity, regrowth capability, and the presence of active nuclei. A decrease in these parameters indicate attainment of maximum life span. In past studies, and in the present one, control cultures are grown on a standard defined medium of four percent glucose concentration. Caloric restriction is accomplished by lowering the concentration of glucose present initially in the medium. Those cultures grown on one of three regimens of dietary restriction are examined for increased longevity in comparison to control cultures based on maintenance of the parameters mentioned above.

MATERIALS AND METHODS

Organism.

Stock cultures of Aspergillus ornatus Raper, isolates of a culture originally obtained from D.J. Schwemmin, were stored in the dark at 4C on 1.5 percent agar slants containing a complex organic medium [36].

Stocks were transferred to fresh slants every two months and parent cultures were made from the stock every four weeks.

Spore Production.

Hyphae were removed periodically from parent cultures and innoculated onto complex medium agar slants. The subcultures were allowed to grow for 72 hours in the dark at 22C, then exposed to light (a combination of fluorescent and tungsten light provided maximum conidia production) for 24 hours. After the exposure period, the slants were returned to the dark for an additional 72 hours. This procedure resulted in dense sporulation.

Spraying Procedure.

Spores were harvested by adding sterile distilled water to the sporulating cultures, which were then agitated on a Vortex-Genie (Scientific Instruments, Springfield, Mass.) for 15 seconds. The spore solution was aseptically collected and decanted into a sterile centrifuge tube and centrifuged for 10 minutes at 12,000 rpm at 24C in a Sorvall RC-5B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) The pellet was washed in sterile distilled water then centrifuged once more. The spores were resuspended in sterile distilled water to an optical density of 0.8 at a wavelength of 450nm as measured by a Spectronic 20 colorimeter (Bausch and Lomb, Rochester, N.Y.)

The resultant spore suspension was sprayed using a sterilized chromatograph sprayer (Sigma, St. Louis, MO.) onto 75 ml of defined medium [30] in 100 ml petri plates previously covered with sterile 0.4u polycarbonate membranes (Nucleopore Corp., Pleasanton, CA.). The air needed to provide the pressure for the sprayer was sterilized by passing it through a Millex FG50 0.2u filter unit (Millipore, Molsheim, France). The spray was delivered twice approximately 20 cm from the surface for one second duration bursts to insure all areas of the membrane surface were covered evenly. The

entire spraying procedure occured in a glove box located in a UV irradiated room. After spraying, the plates were immediately covered and placed in a dark incubator at 22C until sacrificed.

Nutritional Media.

Four nutritional levels were studied. The composition of each regimen consisted of defined media with a variation in glucose concentrations. Control groups (C) were grown continuously on a medium initially containing 40g/L glucose; slightly restricted groups (SLR) on 30g/L glucose; moderately restricted groups (MR) on 20g/L glucose; and severely restricted groups (SR) on 10g/L glucose. The glucose concentration present initially was allowed to become depleted in order that dietary restriction could take place after a mature mat was formed.

DNA Extraction and Assay.

Mats were removed from membranes at 24 hour intervals from 96 to 192 hours, 240 hours, and 336 hours after spraying, quick frozen with liquid nitrogen and stored in 10 ml vials at -52C in a Cyro-Frig C-750

freezer (American Scientific Products, McGaw Park, Ill.) until time of disruption.

Frozen mats were placed in 75 ml glass disrupter flasks (VWR #34007-066, VWR Scientific, Baltimore, Md.) with 50 gm of 0.45-0.55 mm glass beads (VWR #34007-146) and 15 ml of 0.05M phosphate buffer (pH 7.0). The material was homogenized for 60 seconds under continuous gas-liquid CO cooling on a Braun MSK mechanical cell disrupter. The homogenate was pipetted into 3 ml lypholizing tubes and freeze dried for 6 to 8 hours on a Virtis Model 3 Series freeze dryer (The Virtis Co., Inc., Gardiner, N.Y.) at -35C and 100 millitorr. The dried fungal powder was removed from the tubes and stored in 10 ml vials at -52C until time of assay.

Seventy five milligrams of dried powder was suspended in 10 ml of cold (4C) 5 percent trichloroacetic acid (Fisher Scientific, Pittsburg, PA.) for 12 hours. Centrifugation (12,000 rpm at 4C for 10 minutes) and resuspension into cold 5 percent TCA was performed three more times for 12, 4, and 2 additional hours. Two 1 hour incubations in a 3:1 (v/v) 75 percent ethanol-ether solution followed the last incubation in cold TCA. The suspension was centrifuged in between the ethanol-ether incubations. The pellet was resuspended in 2 ml of 5 percent TCA and boiled for 30 minutes in a

water bath. The boiled solution was then sedimented at 15,000 rpm for 10 minutes and the supernatant decanted and saved for assay. The supernatant (0.05 ml) was mixed with 2 ml of diphenylamine reagent [37] and heated for 10 minutes in a water bath (>93C). The intensity of the blue color produced was read at 600nm on a Zeiss PMQII Spectrophotometer for DNA content [37]. Because of interference caused by incomplete removal of carbohydrates (producing a green color), a reading at 650nm was taken and the DNA content expressed as a net optical density [38]. A standard curve was prepared relating optical density to micrograms of DNA, with purified DNA as the standard (Appendix I).

Glucose, pH, and Dry Weight Determination.

Glucose remaining in the spent defined medium after specified periods of fungal growth was measured by boiling 1.0 ml of 3,5-dinitrosalicylate reagent [39] with 0.5 ml of 1.5 percent agar solution (diluted 9:1) for 5 minutes in a water bath. After boiling, the solutions were cooled and 18 ml of distilled water added. The optical density of the solutions was read at 540nm on a Zeiss PMQII Spectrophotometer [39]. A standard curve was prepared relating optical density to

percent glucose with a serial dilution of a 10 percent glucose solution (Appendix I).

The pH of the spent defined medium was measured using an Accumet pH meter (Fisher Scientific, Pittsburg, PA.) by the immersion of the electrode (Fisher Scientific #13-639-252) directly into the agar.

Dry weight of the fungus was determined by the removal of 2 disks (1.0 cm) from each mat using a cork borer. After drying at 55C for 24 hours in ceramic crucibles, the disks were weighed on a XA Analytical Balance (Fisher Scientific).

Induction and Measurement of OPCA Carboxylyase.

For the enzyme induction of OPCA carboxylyase of MR, SLR, and C cultures, mycelial mats of 144 and 336 hours growth were removed from their membranes and floated on 10 ml of liquid defined induction medium containing 0.10 percent L-tryptophan. The mats were returned to the dark to incubate for 6 hours at 22C. SR cultures were removed at 144, 336, and 432 hours of growth and induced for OPCA carboxylyase as above.

To discount the possibility that low activity levels were due to depletion of nutrients needed to synthesize the enzyme, a recovery experiment was carried

out by transferring SR, MR, SLR, and C 336 hour cultures onto 20 ml of fresh solid 4 percent defined medium (without L-tryptophan) and allowing them to incubate for 6 hours in the dark at 22C. After the incubation period, enzyme induction was performed as mentioned previously.

At the end of the induction period, the mats were quick frozen with liquid nitrogen and stored at -52C until time of assay. Determination of activity of OPCA carboxylyase was made by homogenizing 2 gm of the fresh weight of each mat in a 75 ml glass disrupter flask with 15 ml of 0.05M phosphate buffer and 50 gm of 0.45-0.50 mm glass beads. Disruption was done in a Braun MSK cell homogenizer for 60 seconds while being continuously cooled by a gas-liquid CO stream to inhibit heat inactivation of the enzyme. After disruption, the flasks were placed in a 4C refrigerator and the beads were allowed to settle for 10 minutes. The homogenate was poured into a centrifuge tube and spun at 10,000 rpm for 10 minutes. The resultant supernatant represented the crude enzyme homogenate.

The reaction mixture for the determination of enzyme activity was composed of a 1.0 ml sample of an enzyme preparation; 1.0 ml citrate-phosphate buffer (0.1M-0.2M) adjusted to pH 5.2; 0.2 ml distilled water;

and 0.2 ml 0.01M OPCA. An inactivated blank was prepared by substituting the 0.2 ml of water with 0.2 ml of 5 percent HgCl. The reaction was allowed to proceed for 20 minutes in a constant temperature water bath at 30C and stopped with the addition of 1.0 ml of 1N HCl. The OPCA remaining was extracted with 10 ml of ethyl acetate and the phases separated at 4,000 rpm for 5 minutes in a clinical centrifuge (International-C1, Needham Heights, Mass.). The disappearance of OPCA compared to that of the inactivated blank was measured at 320nm on a Zeiss PMQII Spectrophotometer [27]. A standard curve was prepared relating optical density to nanomoles of OPCA (Appendix I).

Protein content of the crude enzyme homogenate was determined using a modification of the biuret method of Gornall [40]. To 1.0 ml of the homogenate was added 4.0 ml of biuret reagent [40] and allowed to react for one hour. The samples were centrifuged at 15,000 rpm for 5 minutes to sediment any cloudy percipitate. The color change was measured at 540nm on the Zeiss PMQII Spectrophotometer. A standard curve was prepared relating optical density to milligrams of protein, using bovine serum albumen as the standard (Appendix I).

One unit of specific activity of OPCA carboxylyase is defined as that amount of enzyme which catalyzes the

disappearance of one nanomole of substrate per minute per milligram of protein [28].

Racetube Studies.

Glass tubing (1.0cm in diameter) was cut into 12 inch pieces and then bent at 45 degree angles 1.5 inches from each end. The racetube ends were covered with metal caps and sterilized. Five milliliters of sterile complex organic medium was syringed into each tube and allowed to incubate in the dark for 48 hours to insure sterility. Innoculations were made at one end of each tube with 5 mm disks cut from mats and inserted into the tube with a sterilized spatula. Growth in the racetubes was measured every 48 hours (for a total of 336 hours) underneath a dissecting scope (Bausch and Lomb, Rochester, N.Y.) and returned to a dark incubator at 22C after each measurement.

Microscope Studies.

Five millimeter disks were removed from 144 and 336 hour mats of each group using a cork borer and placed in a formaldehyde-glutaldehyde fixing solution (0.167M-0.25M) for 6 to 8 hours. After fixation, disks

were rinsed in McIlvaine's pH 4.0 buffer [35] for three 15 minute washings, and stored in the buffer at 4C, until time of observation.

The staining protocol for DNA is a 1:1 (v/v) mixture of 1 g/ml solution of 4'-6 diamidino-2-phenolindole (DAPI) and McIlvaine's buffer. A small section was removed from each disk and shreaded with 25 gauge needles under a dissecting scope. The fungal cells were examined using an Olympus BH-2 epifluorescence microscope (Olympus Optical Co., LTD, Tokyo, Japan) at a wavelength range of near-UV light (340-380nm) [35].

Statisical Analysis.

The results are presented as mean plus or minus standard error, and the significance of differences evaluated by a oneway analysis of variance. The analysis used the procedures of SPSSX Version 2.1 (College of William and Mary, Williamsburg, VA.). When the F-value was found to be significant, the Student-Newman-Keuls multiple range test was used to determine differences in mean values between two groups.

RESULTS

Growth of Organism.

Growth is generally considered to be an increase in the mass of an organism. With respect to fungus, weighing of the dried organism is the most widely used and most convenient method of measuring growth. Dry weight for the varying concentrations of glucose are described in table 1 and figure 1. C and SLR groups showed the most rapid increase during the initial 144 hours of growth, with continued increase in weight with age, obtaining a maximum dry weight of 5.8 $\pm$ .10 mg for SLR groups and 7.2 $\pm$ .20mg for C groups at 336 hours of growth. A oneway analysis of variance determined significant differences in the dry weights of 144 hour ($F=45.6$, $p<.001$) and 336 hour ($F=70.2$, $p<.001$) cultures of the different groups. The dry weights of MR and SR groups were significantly less than those of the other two groups for the first 144 hours of growth, as determined by the Student-Newman-Keuls multiple range test ($p<.05$). There was no increase in dry weight with progressing age in MR and SR groups (Student's t-test, $p>.05$).

Estimation of cell number by quantitative measurement of DNA is illustrated in figure 2. All groups reached a DNA plateau at 120 hours of growth

based on a change in the slope of increasing DNA content, denoting the shift from the log phase to the stationary phase of growth. The results were similar to those of previous studies (Spiegleman and Coursen, 1975; Wilson and Coursen, 1986). The DNA plateaus were analyzed by a oneway analysis of variance which revealed significant differences in the total amount of DNA present in the stationary phase of the different groups ($F=94.9$, $p<.001$). C and SLR groups achieved significantly higher DNA plateaus than MR and SR groups, as determined by the Student-Newman-Keuls multiple range test ($p<.05$). The gradual increase in DNA of C and SLR groups in the late stationary phase is due to the wrinkling and uplifting of the mycelial mat, which allows production of new vegetative tissue on the underside. MR and SR groups show an increase in DNA content at 336 hours of growth due to the occurrence of sporulation. There was no wrinkling present in these groups.

Measurement of glucose remaining in medium used to support the growth of the fungus revealed a steady decrease in glucose with age. All groups utilized $7.0 \pm .35$ g/L of glucose in the first 144 hours of growth. By 336 hours, restricted groups had completely depleted the medium of glucose and were apparently

utilizing other nutrients in the medium as a source of carbon.

The pH of the growth medium decreased steadily in C and SLR groups from an initial value of 5.00 units to 4.00 $\pm$ .01 and 4.24 $\pm$ .02 units, respectively. Most of the drop in pH occurred prior to 96 hours and after 192 hours of growth. The growth medium of MR groups maintained a constant pH of 4.42 $\pm$ .03 units in the stationary phase while SR growth medium showed a decrease in pH from 5.00 units to 4.56 $\pm$ .03 units at 144 hours, followed by an increase in pH, surpassing the initial pH by 240 hours of growth and continually increasing with age.

When the media and membranes which had supported growth for 336 hours when reinoculated with young hyphae from stock cultures, growth was retarded. However, the spent medium was still able to support growth for 192 hours, which suggested that neither pH nor the absence of detectable glucose served to inhibit growth processes.

Racetube Studies.

How readily the organism could adapt to a new environment was determined by studying the different

phases of growth in a glass tube filled with 4 percent complex medium. Growth rate was defined as the amount of hyphal extension per 48 hours. The amount of growth that took place in the first 48 hours after inoculation represented the lag phase of growth. The next 48 hours (48-96 hours) represented the log phase of growth. The stationary phase was comprised of the time period of 144 to 336 hours, with measurements taken every 48 hours to determine the growth rate.

Comparison of the young 144 hour groups revealed no significant differences in either the lag phase ($F=0.74$, $p=.54$), the log phase ($F=1.77$, $p=.19$), or the stationary phase ($F=.035$, $p=.79$) of growth, based on a oneway analysis of variance of growth rates (Fig. 3).

Significant differences were present in all three phases of growth in old 336 hour groups as determined by a oneway analysis of variance of growth rates (table 2). SR cultures had a significantly higher growth rate in the lag phase than any of the other groups (SNK multiple range test, $p<.05$). Analysis of the log phase showed significantly greater growth rates in MR and SR groups compared to that of SLR and C groups (SNK multiple range test, $p<.05$) as illustrated in figure 4. Growth rate comparisons between MR and SR groups and between SLR and C groups revealed no significant differences at the 0.05

level (SNK multiple range test).

The pair wise grouping of MR and SR cultures and SLR and C cultures based on significant differences in growth rates were present in the stationary phase as well. MR and SR groups attained a significantly greater stable growth rate in the stationary phase than SLR and C groups (SNK multiple range test, $p<.05$).

These results led to the study of senescent 432 hour SR cultures which revealed a decrease in the growth rate does occur with further aging of the culture. Racetube studies showed no significant difference in either the lag phase ($F=1.04$, $p>.30$) or the stationary phase ($F=0.33$, $p>.70$) of growth in 144, 336, and 432 hour SR groups, as determined by a oneway analysis of variance. However, there did exist a significant difference in the log phase between the different age groups ($F=41.8$, $p<.001$). The log phase of 432 hour SR groups was significantly different from that of 144 and 336 hour groups (SNK multiple range test, $p<.05$), and represented an intermediate growth rate between that of 144 and 336 hour SR groups (Fig. 5).

Figures 6 and 7 illustrate the cummulative growth of the organism. The old 336 hour cultures of SR and MR groups attained significantly greater total growth than SLR and C groups of the same age (SNK multiple range

test, $p<.05$). Similarities in the growth rate of all young 144 hour groups resulted in similar cummulative growth, as determined by a oneway analysis of variance ($F=0.73$, $p>.50$)

Microscope Studies.

To determine if the differences in dry weight and regrowth capability of the diet groups was due to the amount of nuclear material present, cultures were examined for DNA content. DAPI bound to DNA produced a blue fluorescence. Examination of hyphae revealed the presence of active nuclei in young 144 hour and old 336 hour cultures of all groups, and scenescent 432 hour SR groups (Plates 1 and 2). Due to a lack of a microspectrofluorometer, a quantitative determination of the amount of light emitted from a particular region of fluorochrome stained DNA could not be made (Coleman et al., 1981).

The presence of yellow fluorescent bodies in unstained 144, 336, and 432 hour SR groups suggest the release of polyphosphates from the cell wall into the hyphal lumen (Coleman, 1978) as illustrated in plate 3. Polyphosphates were not present in 144 and 336 hour C cultures (Plate 4).

Examination of unstained material revealed that A. ornatus is autofluorescent, producing a green fluorescence when excited at a wavelength range of near UV-light.

OPCA Carboxylyase Activity.

The basal activity for the enzyme was found not to be significantly different in the young 144 hour cultures of the different diet groups by a oneway analysis of variance ($F=1.03$, $p=.42$). There was a decrease with age in basal activity levels for all groups (Student's t-test, $p<.05$). The amount of decrease in activity was found to be significantly different between the diet groups (oneway analysis of variance, $F=21.9$, $p<.001$). Old 336 hour cultures of SR and MR groups maintained greater basal activity levels than old SLR and C cultures of the same age (SNK multiple range test, $p<.05$). A further increase in age of SR groups did not result in a continuous decline of basal activity as indicated by similar activity levels of 336 and 432 hour cultures (Student's t-test, $p>.05$).

The effect of age and nutrition on the level of activity to which OPCA carboxylyase can be induced is shown in figures 8 and 9, and table 3. Specific

activity of the enzyme was analyzed by a oneway analysis of variance which showed a highly significant effect of nutrition on both "young" 144 hour cultures ($F=29.2$, $p<.001$) and "old" 336 hour cultures ($F=159.9$, $p<.001$). Using the Student-Newman-Keuls multiple range test, it was shown that the specific activity of OPCA carboxylyase in induced MR and SR groups was significantly lower ($p<.05$) than that of induced SLR and C groups at 144 hours of growth. There was no significant difference in specific activity between induced MR and SR groups ($12.57 \pm .30$ and $11.97 \pm .29$ units, respectively) and between induced SLR and C groups ($15.83 \pm .43$ and $15.98 \pm .50$ units, respectively) at the 0.05 level (SNK multiple range test).

In the old 336 hour induced cultures of MR and SR groups, specific activity levels were similar to those determined for the young age induced cultures of MR and SR groups (SNK multiple range test, $p>.05$). On the other hand, the specific activity of old 336 hours induced cultures of SLR and C groups were on the average 62 percent lower than their counterparts at 144 hours of growth (SNK multiple range test, $p<.05$).

The results of the recovery experiment are represented in figure 10. There was no significant difference in activity between 336 hour induced cultures

and those cultures placed on the recovery medium prior to induction in MR, SLR, and C groups (SNK multiple range test, $p>.05$). However, SR groups placed on the recovery medium prior to induction showed a slight increase in specific activity in comparison to 336 hour induced SR groups (SNK multiple range test, $p<.05$).

Based on induced levels of OPCA carboxylyase specific activity, it was determined that SR groups exhibited a 22 percent increase in longevity compared to C groups. Senescent 432 hour SR cultures had significantly lower OPCA carboxylyase specific activity than 336 hour SR cultures (T-test, $p<.05$). The crash in activity, though at a slightly lower level, resembled that of 336 hour C group (T-test, $p<.05$), as illustrated in figure 11.

Correlation coefficients were calculated to determine associations between growth (as determined by dry weight) and OPCA carboxylyase activity. The correlation coefficient did not reach statistical significance ($r=.80$, $p>.05$) in young 144 hour cultures. In contrast, there existed a significant positive correlation of depressed growth and greater enzyme activity in old 336 hour cultures ($r=.949$, $p<.01$).

Protein Levels.

Since the specific activity of OPCA carboxylyase is based directly on total protein, protein levels in the crude homogenate were measured. The data shown in table 4 represent total protein present in the crude homogenate with age. A oneway analysis of variance determined that a significant difference in protein levels existed at the different ages, both within and between groups ($p<.001$). The Student-Newman-Keuls multiple range test was used to determine groups significantly different at the 0.05 level.

Basal protein levels were significantly lower in SR and MR groups in comparison to SLR and C groups at all ages tested ($p<.05$) as illustrated in figure 12. Total protein decreased with age in SR and MR groups, while increasing in SLR and C groups (Student's t-test, p,>05). The change in the amount of total protein in 336 hour cultures was significantly different between all diet groups (SNK multiple range test, $p<.05$). In accordance with the basal level of OPCA carboxylyase activity of SR cultures, total protein did not significantly decrease once old age was attained as indicated by similar protein levels of 336 and 432 hour SR cultures (Student's t-test, $p>.05$). The results are illustrated in figure 14.

At 144 hours, total protein in induced SR and MR cultures (3.85 $\pm$ .04 and 3.53 $\pm$ .08 mg, respectively, $p<.05$) significantly exceeded induced cultures of SLR and C groups (2.62 $\pm$ .07 and 2.55 $\pm$ .06 mg, respectively, $p>.05$) As in basal cultures, protein levels in induced SR and MR cultures decreased with age, while increasing in induced cultures of SLR and C groups (Fig 13). At 336 hours, protein levels had dropped by 32 percent in induced SR cultures (2.63 $\pm$ .04 mg) and by 10 percent in induced MR cultures (3.18 $\pm$ .04 mg). Induced SLR and C groups of the same age showed an increase in protein levels by 53 percent (5.57 $\pm$ .14 mg) and 61 percent (6.55 $\pm$ .10 mg), respectively.

Total protein continued to decrease with age in induced SR groups as illustrated in figure 14. Senescent 432 hour induced SR cultures had significantly lower protein levels than those of 144 and 336 hour induced SR cultures (SNK multiple range test, $p<.05$).

There was no significant difference at the 0.05 level in protein levels of induced 336 hour cultures and those cultures placed on the recovery medium prior to induction for OPCA carboxylyase for any of the groups as determined by a Student's T-test.

DISCUSSION

It is more than 50 years now since McCay, Crowell, and Maynard [41] first reported that restricting the food intake of rats markedly increased the maximum length of life attained by a rat population. Since these early studies, the increased life span associated with underfeeding has been reported in mice [21,42], in hamsters [43], in rats [44,45], in fish [24], in Daphnia [46], in Drosophila [47], in rotifers [23], and in protozoa [48]. A significance of the present study is the report of increased longevity via dietary restriction in an organism outside of the Kingdom Animalia, giving support to the universality of the effect of reduced food intake on increased longevity. Though measurement of longevity in Aspergillus ornatus cannot be made directly as in mice and rats, the indirect parameters of enzyme activity, regrowth capability, and the presence of active nuclei serve as proven indexes for measurement of the aging process.

Associated with increased longevity in dietary restricted animals is the depression of body weight. Numerous studies of mice and rats show a positive correlation between low body weight and increased longevity [21,45]. Determination of the dry weights in different diet groups of A. ornatus indicate results

which are in accord with the rodent studies (Fig 15).

In SR and MR groups maximum dry weight was reached by 144 hours of growth establishing a plateau through the remainder of the life span. In the case of SLR and C groups, there was a steady increase in dry weight with age with peak weight occurring near the end of the life span. At young and old ages, SLR and C groups consistently outweighed SR and MR groups. These results indicated a significant positive correlation between depressed weight and longevity in A. ornatus.

The ability of an organism to promote cellular proliferation when provided optimal conditions gives an indication to its longevity. In a pioneer series of experiments, Lesher, Fry, and Kohn [51] showed that the length of the cell cycle of duodenal epithelial cells increased in aging mice. In young and middle aged mice (93 and 372 days old, respectively) the length of the cell cycle was 11.5 hours, but in old mice (940 days old), it increased to more than 15 hours. The increase in cell cycle time with age was also discovered in cells of the jejunum and ileum [52]. Ryan et al [34] showed that when fungal hyphae were inoculated into a growth tube the rate of growth in the lag and log phases decreased with increasing age of the inoculum. The decrease in the rate of growth was attributed to a

decrease in the rate of synthesis of enzymes needed to metabolize available nutrients, and the rate at which assimilated material was utilized in growth. Comfort [24] showed that caloric restriction not only increased the life span of the guppy (Poecilia reticulata), but also the rate of regeneration of the tail web is greater in restricted fish than in controls at all ages tested [53].

Racetube studies performed on old 336 hour cultures of A. ornatus show a significant difference in the growth rate of the log phase and stationary phase of dietary restricted groups and control groups. SR and MR cultures had significantly greater growth rates than SLR and C cultures. This indicates that aged dietary restricted cultures (MR and SR) reached not only a steady rate of cell proliferation more rapidly than control groups of the same age, but also a higher plateau of stationary growth. The differences in the growth rates were reflected as well in the total amount of growth attained after 336 hours of growth.

Earlier studies carried out in rats [49] and in rotifers [23] indicate that age changes in enzymatic activities occurred later in life of animals whose longevity was increased by dietary restriction. Ross [49] showed that rats on a restricted diet had

maximal ATPase activity at 600 days, while those rats fed ad libitum reached maximal expression at 200 days. Similarly, Fanestil and Barrows [23] showed activity levels of lactic dehydrogenase and malic dehydrogenase were maximum at 15 days in control animals, while at maximum at 36 days in restricted animals. Further studies by Ross [50] revealed consistently lower enzyme activities in young restricted animals compared to young control animals. This fact suggests that reduced enzyme activity at young age may lead to sustained or slightly greater levels of enzyme activity as age increases. In each study, dietary restricted animals had significantly longer life spans than control animals.

Based on the parameter of enzyme induction as seen in OPCA carboxylyase activity, dietary restricted cultures of A. ornatus showed a significant increase in life span compared to control cultures. As the severity of dietary restriction increased, so did longevity.

Using the same parameter, those cultures grown on the control defined medium (group C) attained a life span of 336 hours. In comparison, cultures grown on slightly restricted amounts of the control defined medium (group SLR) showed a 20 percent greater enzyme activity at the same age. The most severly restricted group (SR) showed a 140 percent greater activity than

that of the C group, 100 percent greater than the SLR group, and 16 percent greater than those cultures grown on a moderately restricted diet regimen (group MR) at 336 hours. Ultimately a drastic decrease in activity of OPCA carboxylyase was observed in SR groups, but it occurred 96 hours later than in C groups. Using this parameter, this result represented a 22 percent increase in longevity over that of C groups.

It is important to note that both SR and MR groups had significantly lower enzyme activity than SLR and C groups in young 144 hour cultures. This finding gives support to the notion that lower initial enzymatic activity may result in sustained or even slightly greater levels of enzyme activity with increasing age.

The relationship between depressed total protein synthesis at an early age and increased longevity has been reported in dietary restricted rats [60]. The findings of the present study show a similar relationship. Protein levels of uninduced (basal) cultures of dietary restricted groups (SR and MR) were consistently lower than in control groups at all ages tested. As noted earlier, the restricted groups showed an increase in longevity based on OPCA carboxylyase specific activity.

The elevated levels of total protein in induced

young 144 hour restricted cultures (MR and SR) is thought to be in response to the greatly increased concentration of glucose present in the induction medium as compared to the culture's past history. At 144 hours, the concentration of glucose present in the spent medium of SR and MR groups was down to 3g/L and 13g/L, respectively. The glucose concentration of the induction medium was 40g/L. It is suggested that in response to the new environment, enzymes to assimilate the excess glucose are synthesized resulting in the increase of total protein. The fact that there is a two fold increase in total protein in the dietary restricted groups (MR and SR) by the end of the six hour induction time provides additional evidence that dietary restricted organisms are faster in adapting to a new environment.

Previous studies conducted in this laboratory suggest the existance of a mixture of active and inactive molecules of OPCA carboxylyase in aging cultures [29,59]. It is proposed that the formation of inactive enzymes result in the decreased ability of the enzyme to break down the inducer. The prolonged presence of the inducer allows for continual synthesis of the enzyme [30], both active and inactive forms. Thus, there is a net increase in protein resulting in

decreased enzyme specific activity. Rossiter [29] showed that the amount of antibody precipitated in purified samples of OPCA carboxylyase was twice as much in old 336 hour cultures as in young 168 hour cultures, grown on a medium identical to the control group medium in the present study. Total specific activity of 336 hour cultures was three fold lower than 168 hour cultures. In view of these results, the decrease in OPCA carboxylyase activity in the present study may be attributable to the production of inactive enzymes.

The mechanisms by which altered enzymes may accumulate during aging include changes in amino acid sequence and/or in posttranslational reactions. The likelihood of the latter event being solely responsible for the production of inactive enzymes in this study is discounted due to the overwhelming number of reactions that would have to take place to inactivate such a large number of enzymes. Since growth in A. ornatus is via mitotic divisions, the rise of a mutation can be propagated in the descendents of individual cells. A coding error in the sequence of a particular enzyme due to a mutation in the DNA template could account for the production of a large quantity of inactive molecules.

Efficient DNA repair is generally accepted as essential for maintaining fidelity of the genetic code

and for error-free gene expression. Studies on human fibroblasts (in vitro) show a decrease in the rate of DNA strand rejoining as well as a decreased repair rate with increasing age [54]. Hart et al. [55] and Hall et al. [56] have described a positive correlation between maximum life span of a species and the capacity of fibroblasts and lymphocytes to repair damaged DNA. Recent studies on mice, previously shown to have increased maximum life span when placed on a restricted diet regimen, show that older mice on severe dietary restriction have higher levels of DNA repair than mice of the same sex and age fed on a minimally restricted diet [57]. Yet, the precise mechanisms by which dietary restriction may affect some DNA repair activities are still unknown. A complex interplay between the DNA repair capacity of lymphocytes and their proliferation potential has been suggested [58]. The inability of a cell to enter the mitotic cycle may be due to an accumulation of unrepaired damage.

The use of DAPI in staining for DNA revealed the presence of active nuclei in cell preparations of dietary restricted and control groups at all ages tested. Though the amount of stain associated with the DNA is directly proportional to the amount of DNA present, no quantitative assessment could be made due to

the lack of a microspectrofluorometer. Chemical analysis of the total amount of DNA present in crude homogenates showed that control groups attained greater DNA content than restricted groups (SR and MR). Yet, neither procedure allowed for a qualitative study of DNA (i.e., damaged DNA).

In correlation with the studies on the repair rate of damaged DNA in aging cells mentioned earlier, it is suggested that the decrease in OPCA carboxylyase activity as well as the lower growth rate in racetube studies of control groups may be due to the accumulation of damaged DNA in the genome, resulting in the production of inactive enzymes. In order to fully substantiate this hypothesis, it will be necessary to design a study which would determine whether faulty proteins are produced in dietary restricted cultures, and if so, identify where the error(s) originate. The results of this study suggest that protein levels in dietary restricted groups (MR and SR) should remain stable based on sustained enzyme activity levels with age. In addition, a greater rate of cell proliferation, as measured by total growth, seen in dietary restricted groups (MR and SR) suggest little or no damage to replication mechanisms in the genome of aged cultures.

The low enzyme activity seen in senescent 432 hour

SR cultures may be a result of decreased ability to make OPCA carboxylyase rather than due to the production of inactive enzyme molecules. Associated with decreased enzyme activity in induced 336 hour control groups was an increase in protein content. In contrast, induced 432 hour SR groups do not show an increase in total protein, but in fact, show a significant decrease in the amount of total protein as compared to induced 336 hour SR cultures. The decrease of enzyme activity may be partly due to a reduction in the rate of RNA synthesis, which has been shown to decrease in aging human cells [61].

It is apparent that the duration of the recovery experiment was not long enough to allow for any possible changes in enzyme activity. The use of a solid medium rather than a liquid medium seems to have prevented the rapid uptake of nutrients. Future experiments on recovery capability will need to have an increased incubation period on solid medium, or be carried out on liquid medium.

A current explanation for aging at the cellular level is based on the production of free radicals, with subsequent free radical chain reactions with polyunsaturated fatty acids, nucleotides in DNA, and critical sulfhydryl bonds in proteins. The net result

is a decline in cellular function caused by reduced enzyme activities, error-prone nucleic acid metabolism, and loss of cell membrane fluidity and receptor alignment.

Parker [62] suggests that increased metabolism results in the generation of greater amounts of oxidants and, consistent with the free radical theory of aging, lifespan may critically depend upon the rate of free radical synthesis and the rate at which they cause oxidative DNA damage. A decrease in the amount of available nutrients may allow for a lower metabolic rate, in turn, reducing the rate of free radical generation.

The role of antioxidants and free radical scavengers in aging has been extensively studied. Superoxide dismutase, a free radical scavenger, has been shown to have decreased activity with increased age in human fibroblasts due to the alteration of the enzyme [11]. Superoxide dismutase has been proven to exist in A. ornatus [63]. It is suggested that may be the level of superoxide dismustase also decreases with age in A. ornatus resulting in the progressive loss of the free radical defense mechanism and the subsequent on set of senescence.

It is postulated that nutritional restriction

extends maximum life span by either reducing the use of the genetic code during early life or reducing the generation of free radicals. The proposed mechanism for promoting longevity in either manner is thought to be due to the depression of metabolic activities in the cell, as denoted by lower specific activity, lower DNA content, depressed dry weight, and lesser protein content in restricted cultures of A. ornatus.

Table 1. Dry weight in mg of 1 cm disks cut out from mycelial mats of different diet groups of Aspergillus ornatus vs time (hours of growth). Values are means ±SEM for n=6 samples in each diet/age group. For group descriptions, see text. Statistical significance of differences between group means was evaluated by the Student-Newman-Keuls multiple range test, which was applied when a oneway analysis of variance indicated significant differences. A Student's t-test determined which values between ages of a group were significantly different. Means not sharing a common superscript letter were significantly different (p=0.05).

TABLE 1

Dry Weight vs Age

Age (hours)	F Value	F Prob.	Diet Group SR	MR	SLR	C
144	45.6	p<.001	4.3 ±0.1[a]	4.5 ±0.5[a]	5.0 ±0.2[b]	6.4 ± 0.3[c]
336	70.2	p<.001	4.2 ±0.2[a]	4.6 ±0.1[a]	5.8 ±0.1[d]	7.2 ±0.2[f]

Figure 1. Dry weight in mg of 1 cm disks cut out from mycelial mats of different diet groups of Aspergillus ornatus vs time (hours of growth). For group descriptions, see text. Each point represents the mean of 6 replicates from 3 separate experiments. The vertical lines are the standard error of the mean.

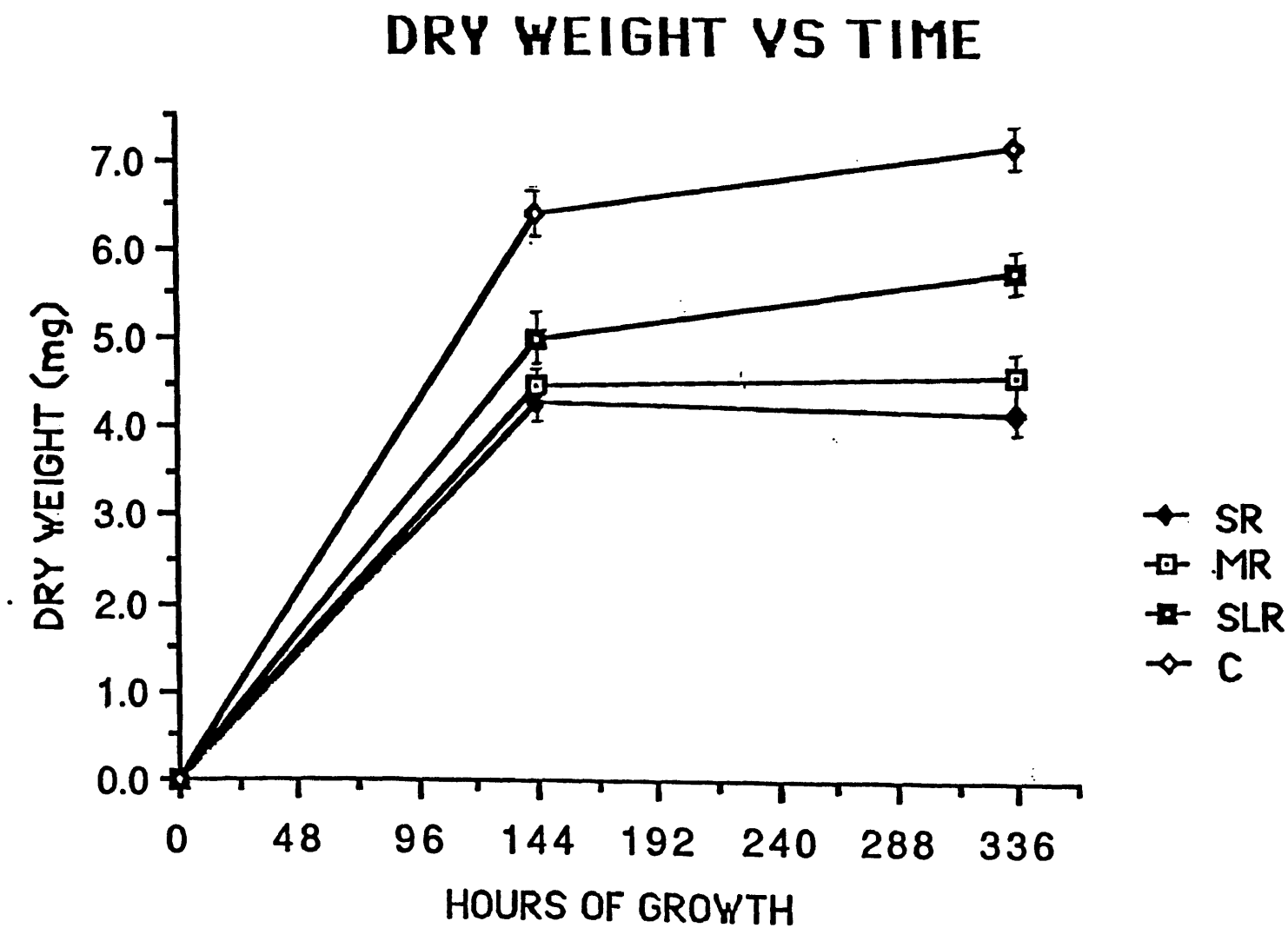

DRY WEIGHT VS TIME
DRY WEIGHT (mg)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
48
96
144
192
240
288
336
HOURS OF GROWTH
SR
MR
SLR
C

Figure 2. Total DNA in mg of different diet groups of Aspergillus ornatus vs time. For group descriptions, see text. Each point represents the mean of 7 replicates from 5 separate experiments.

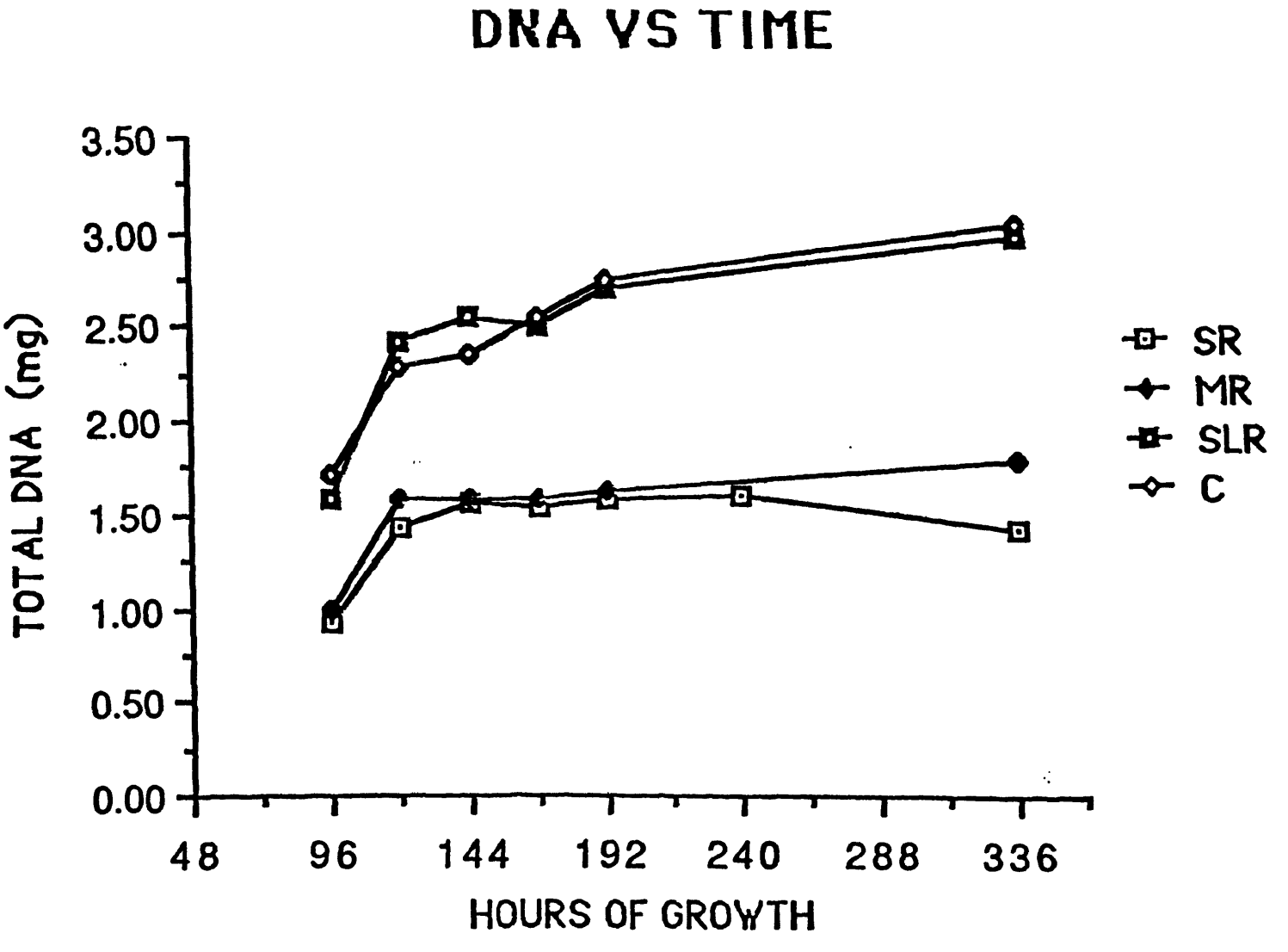

DNA VS TIME
TOTAL DNA (mg)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
48
96
144
192
240
288
336
HOURS OF GROWTH
SR
MR
SLR
C

Figure 3. Growth rate in cm of 144 hour cultures of the different diet groups of Aspergillus ornatus as determined by mycelial progression per 48 hours in racetubes. For group descriptions, see text. Each point represents the mean of six replicates from two separate experiments. The area encompassing the point is the standard error of the mean.

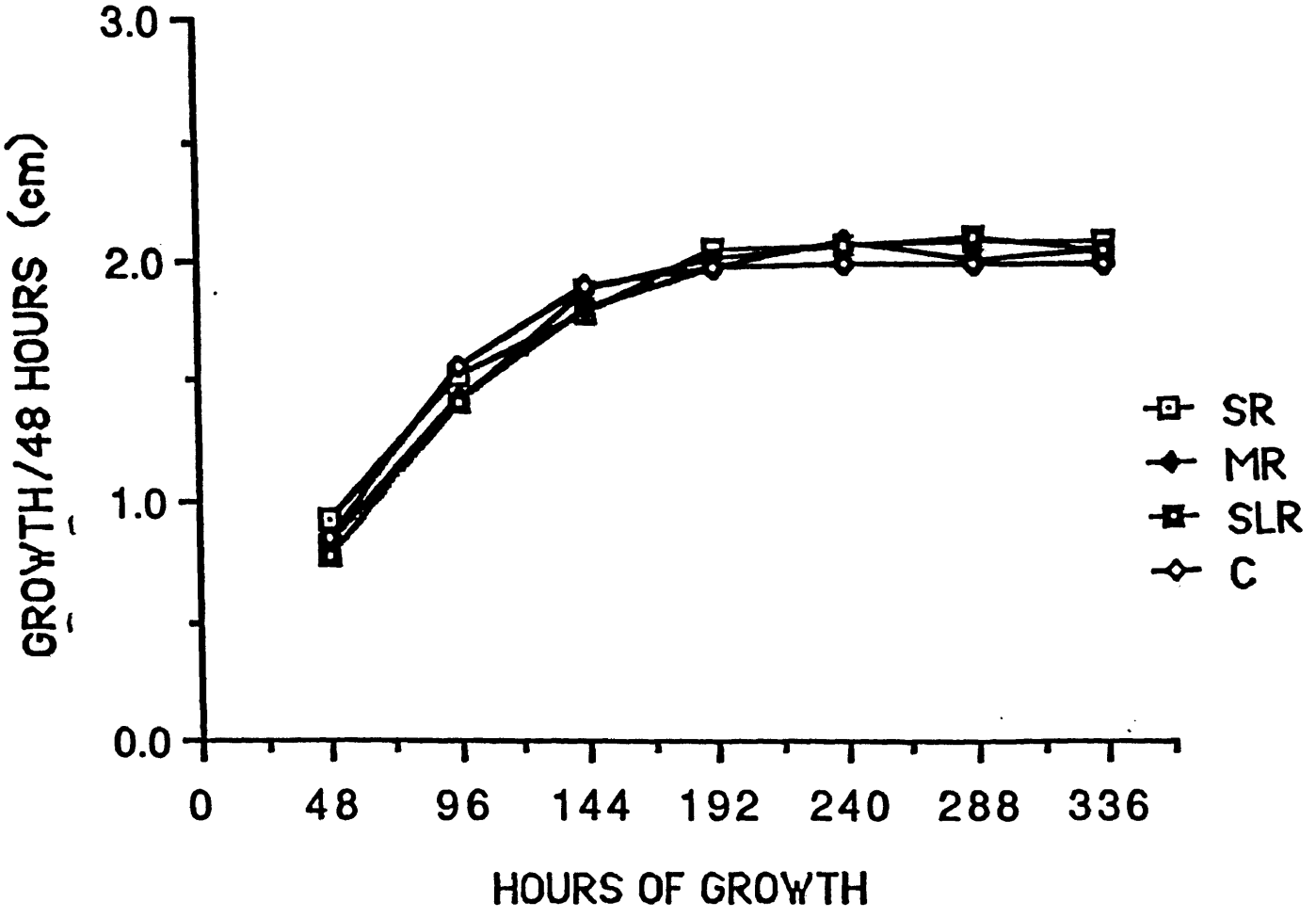

144 HOUR INOCULATE GROWTH RATE
3.0
2.0
1.0
0.0
GROWTH/48 HOURS (cm)
0
48
96
144
192
240
288
336
HOURS OF GROWTH
SR
MR
SLR
C

Table 2. Growth rate in cm of the different diet groups of Aspergillus ornatus vs time. Values are mean ±SEM for n=6 samples in each diet group. For group discriptions, see text. The first 48 hours represents the lag phase. The next 48 hours (48-96 hrs) represents the log phase. The remaining 240 hours represent the stationary phase. Statistical significance of differences between group means was evaluated by the Student-Newman-Keuls multiple range test, which was applied when oneway analysis of variance indicated significant differences. Means in each row not sharing a common superscript letter were significantly different (p=0.05). Means in each column not sharing a common superscript number were significantly different (p=0.05).

TABLE 2
Growth Rate of 336 Hour Inoculates vs Time

Time (hours)	F Value	F Prob.	Diet Group SR	MR	SLR	C
48	4.7	$p<.015$	1.05 ± 0.04^{a1}	0.91 ± 0.05^{b1}	0.87 ± 0.04^{b1}	0.87 ± 0.02^{b1}
96	94.7	$p<.001$	1.98 ± 0.03^{a2}	2.00 ± 0.03^{a2}	1.33 ± 0.05^{b2}	1.38 ± 0.04^{b2}
144-336	19.4	$p<.001$	2.10 ± 0.02^{a3}	2.10 ± 0.01^{a3}	1.97 ± 0.02^{b3}	1.92 ± 0.02^{b3}

Figure 4. Growth rate in cm of 336 hour cultures of the different diet groups of Aspergillus ornatus as determined by mycelial progression per 48 hours in racetubes. For group descriptions, see text. Each point represents the mean of six replicates from two separate experiments. The area encompassing the point represents the standard error of the mean.

336 HOUR INOCULATE GROWTH RATE

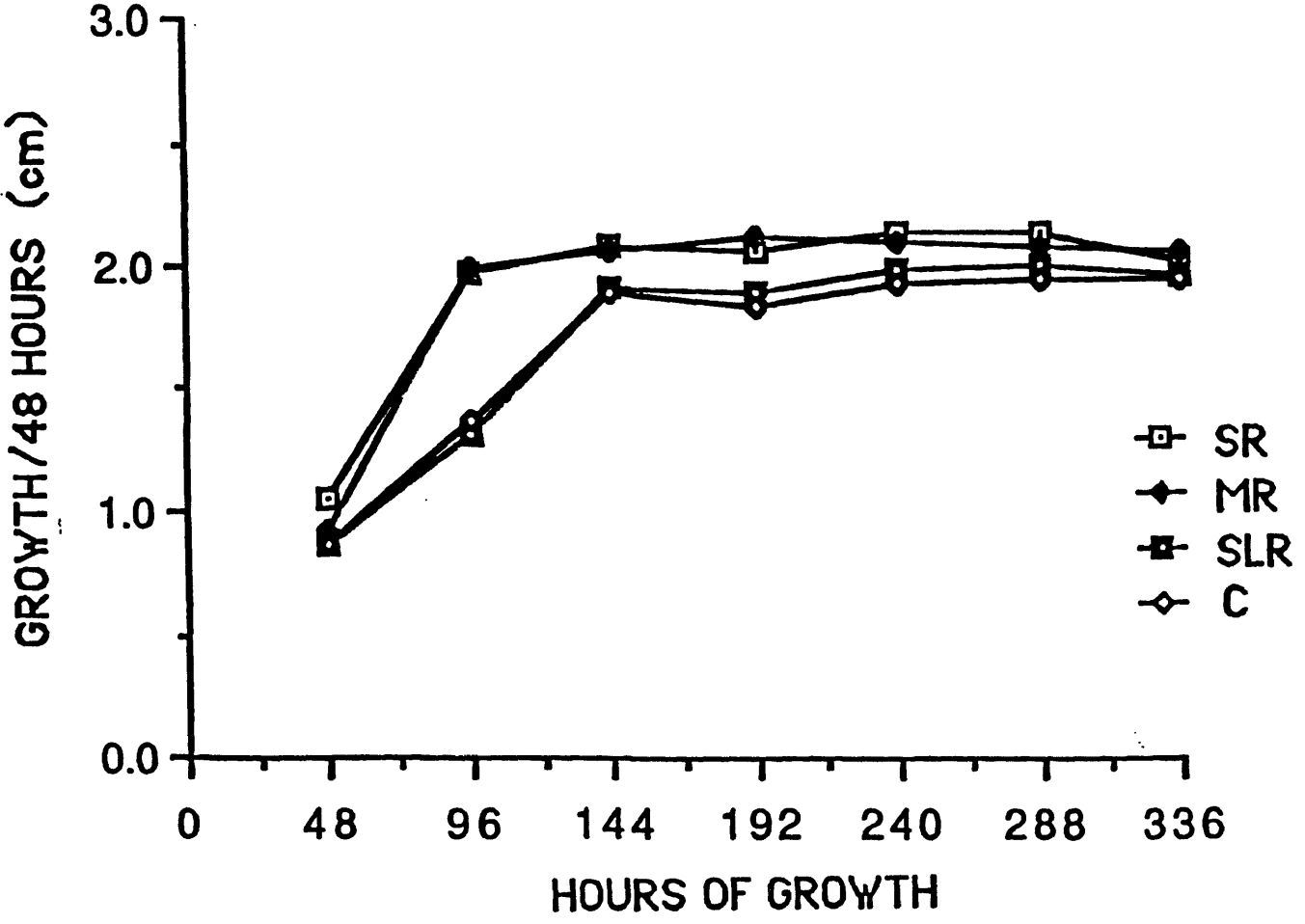


Figure 5. Comparison of growth rates in cm as determined by mycelial progression per 48 hours in ractubes between 144, 336, and 432 hour cultures of the SR group. For group description, see text. Each point represents the mean of six replicates from two experiments. The area encompassing the point represents the standard error of the mean.

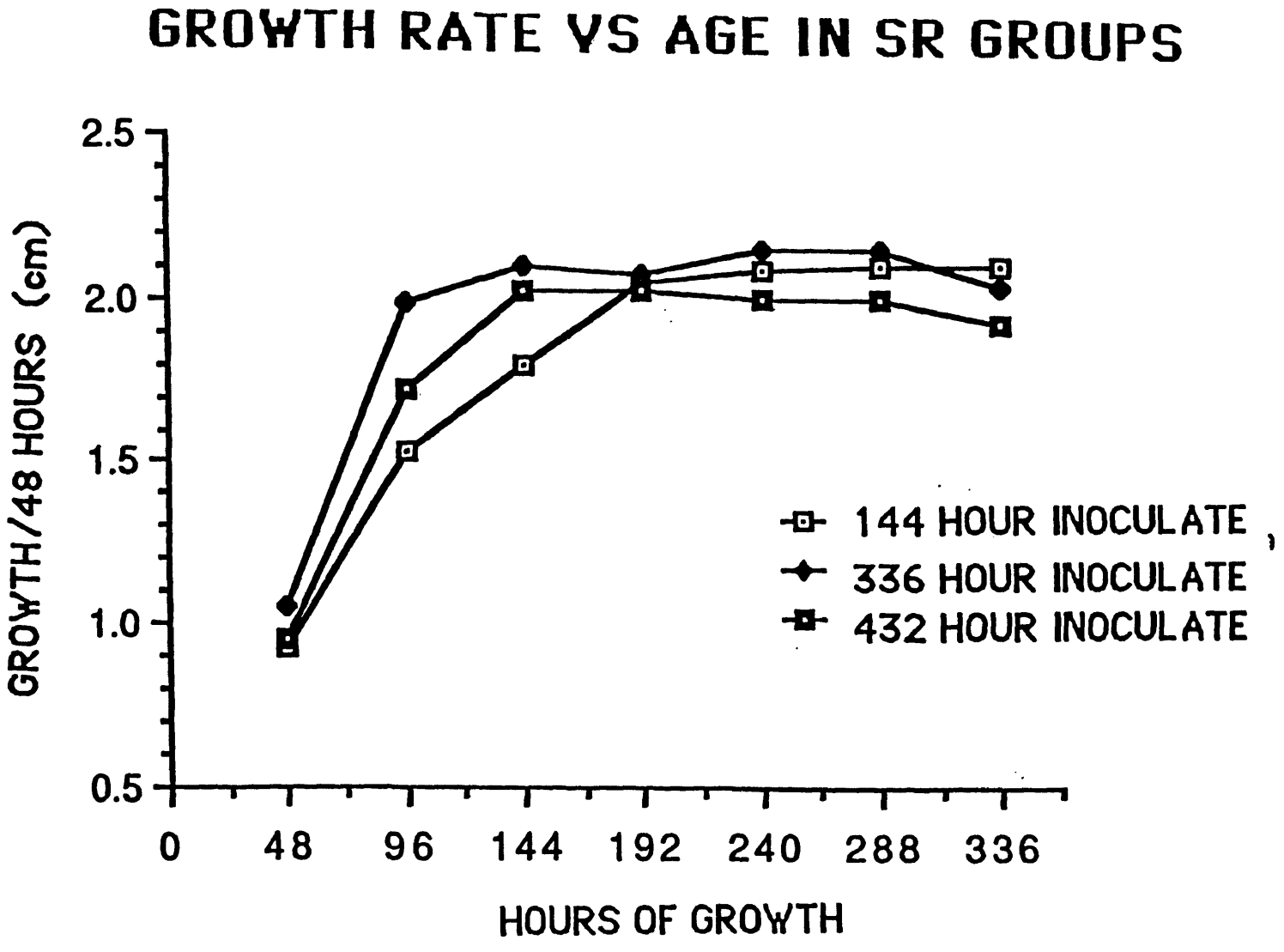

GROWTH RATE VS AGE IN SR GROUPS
GROWTH/48 HOURS (cm)
2.5
2.0
1.5
1.0
0.5
0
48
96
144
192
240
288
336
HOURS OF GROWTH
144 HOUR INOCULATE
336 HOUR INOCULATE
432 HOUR INOCULATE

Figure 6. Cummulative growth in cm of 144 hour cultures of the different diet groups of Aspergillus ornatus as determined by total mycelial progression measured every 48 hours for 336 hours of growth in racetubes. For group descriptions, see text. Each point represents the mean of six replicates from two separate experiments. The area encompassing the point represents the standard error of the mean.

144 HOUR INOCULATE CUMMULATIVE GROWTH

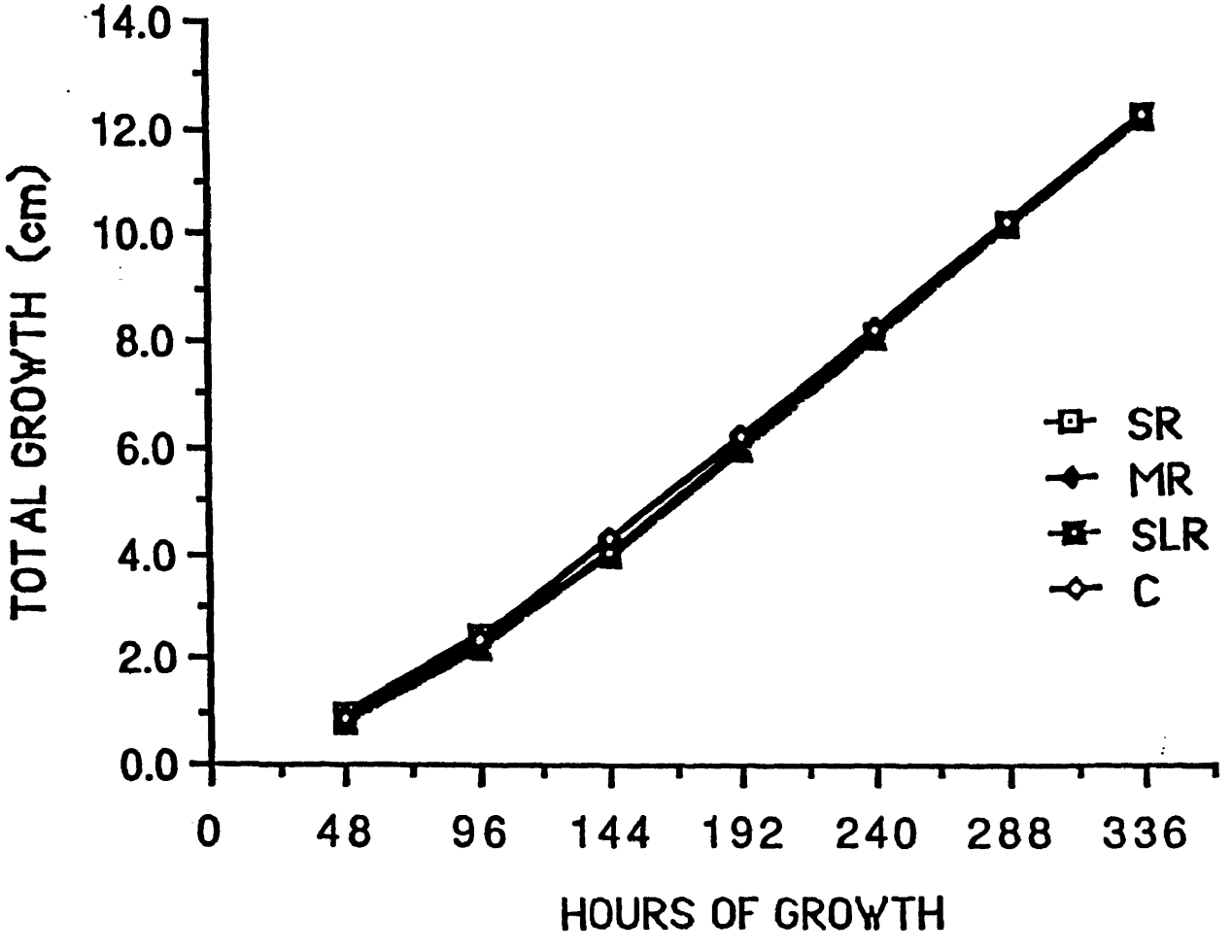


Figure 7. Cummulative growth in cm of 336 hour cultures of the different diet groups of Aspergillus ornatus as determined by total mycelial progression measured every 48 hours for 336 hours of growth in racetubes. For group descriptions, see text. Each point represents the mean of six replicates from two separate experiments. The area encompassing the point represents the standard error of the mean.

336 HOUR INOCULATE CUMMULATIVE GROWTH

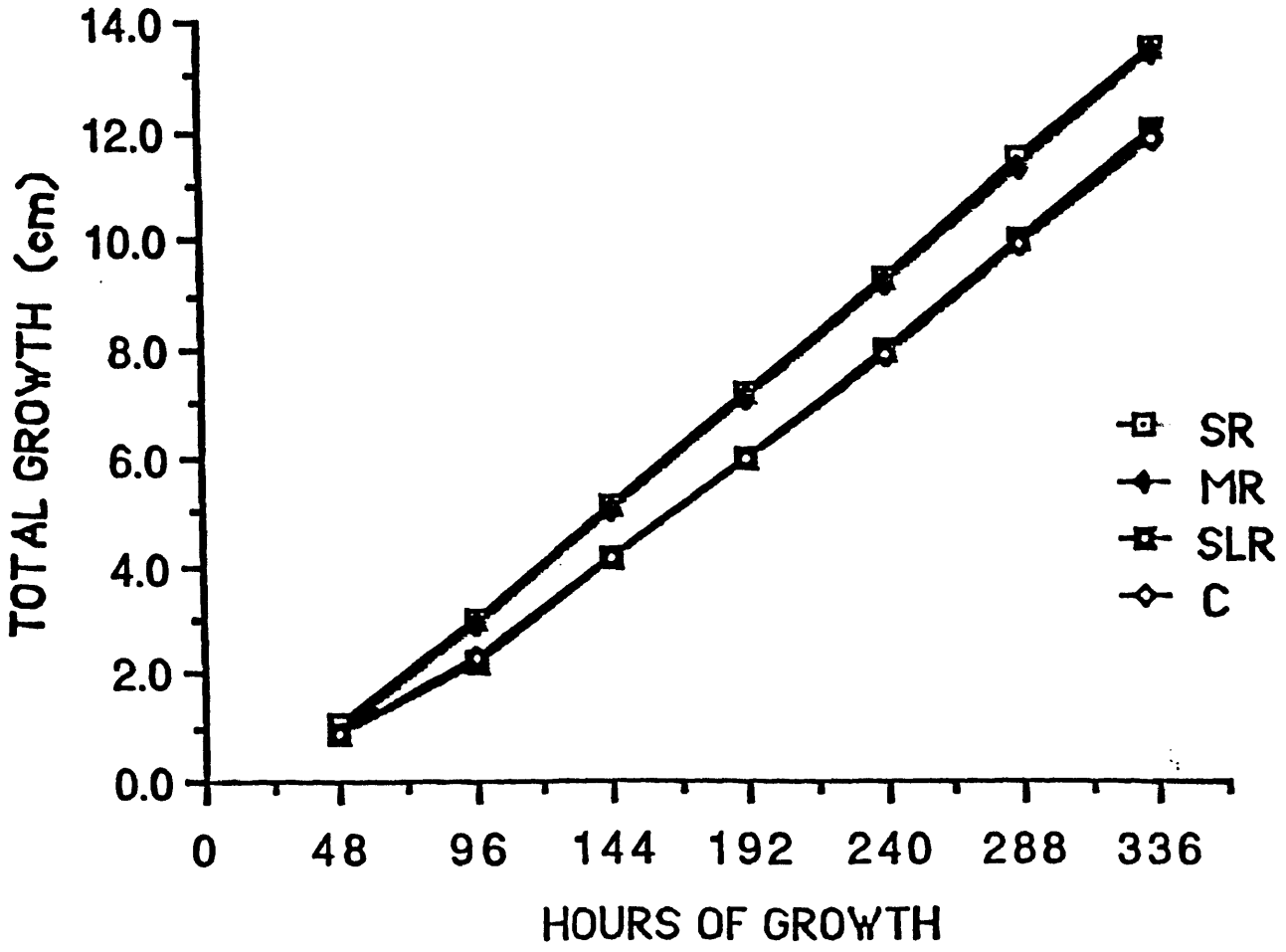


Plate 1a. DAPI stained nuclei of 144 hour SR culture.

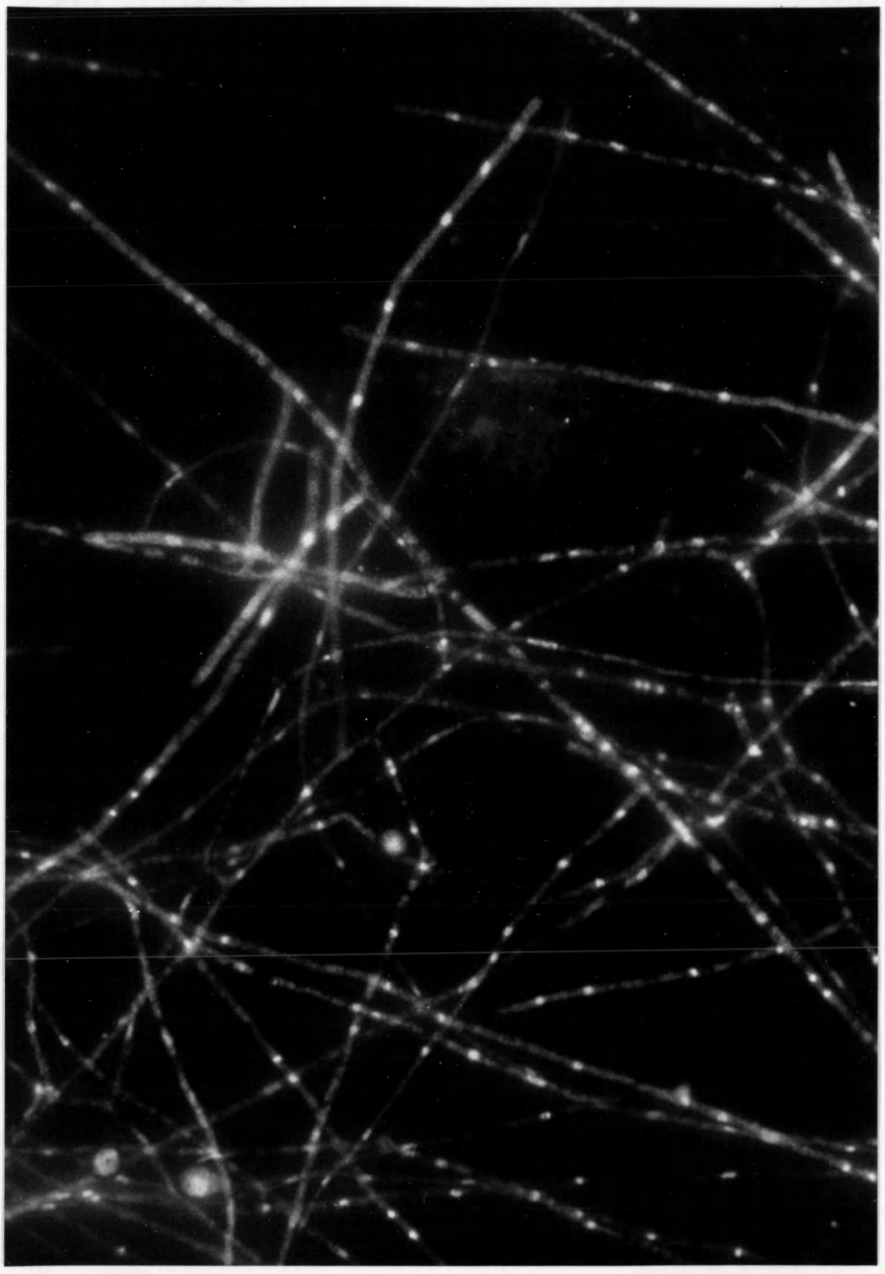


Plate 1b. DAPI stained nuclei of 336 hour SR culture.

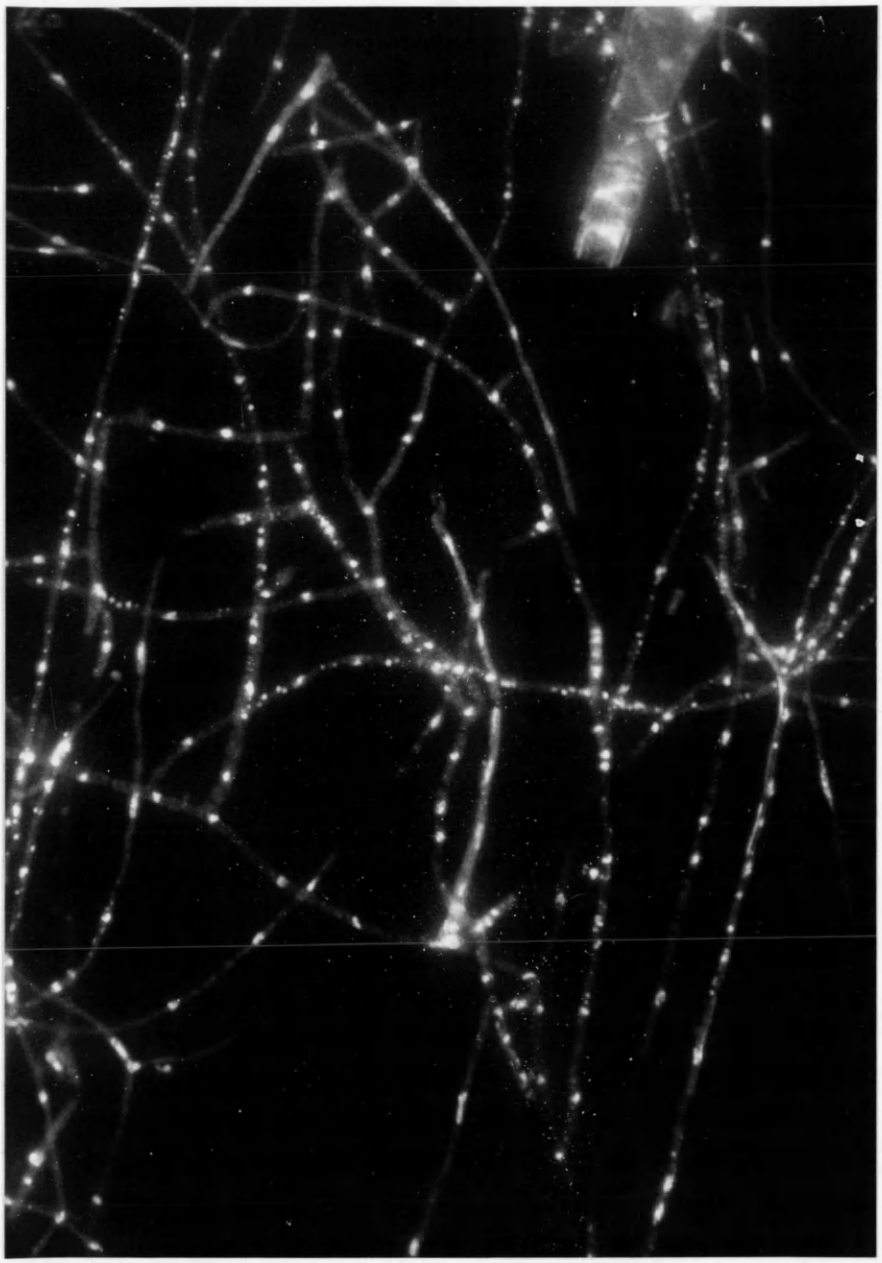


Plate 2a. DAPI stained nuclei of 144 hour C culture.

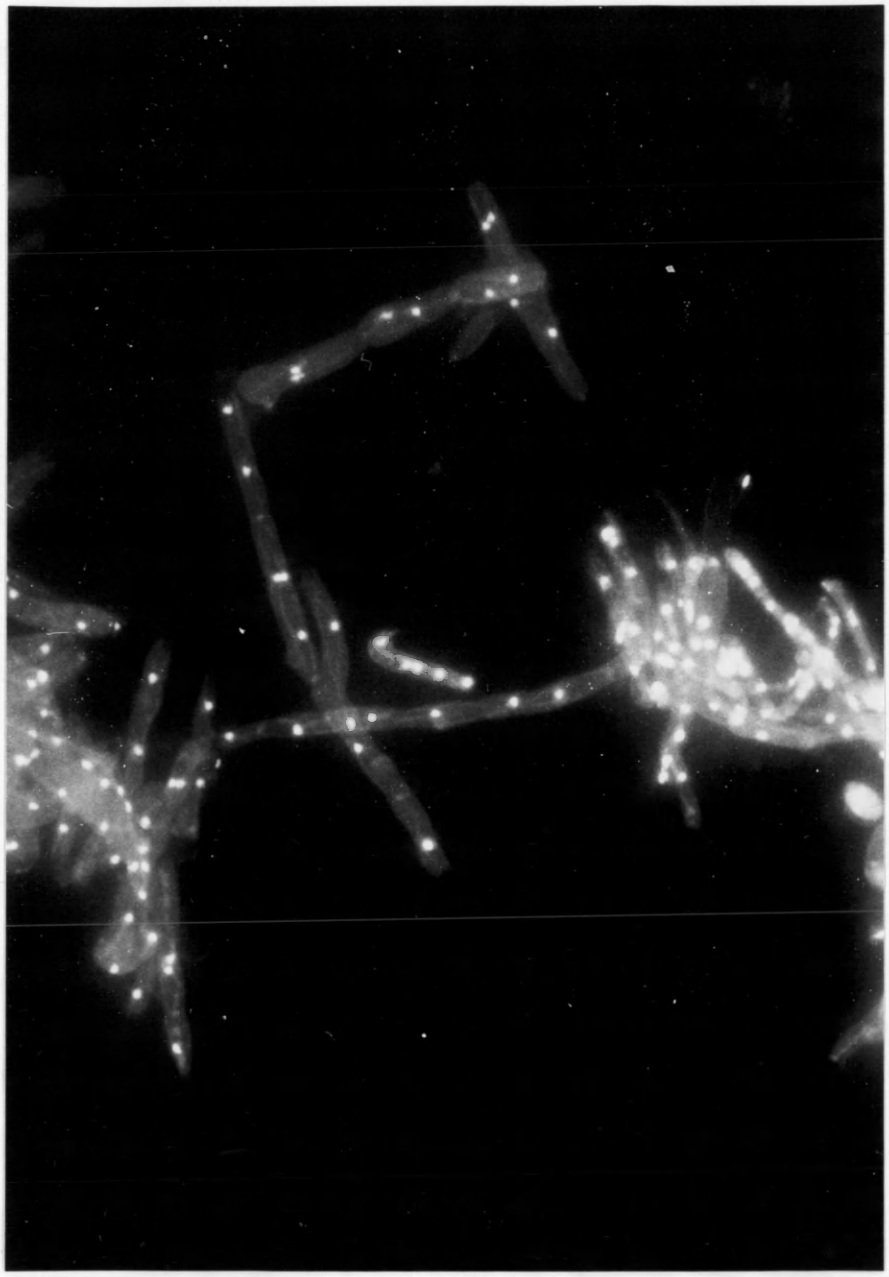


Plate 2b. DAPI stained nuclei of 336 hour C culture.

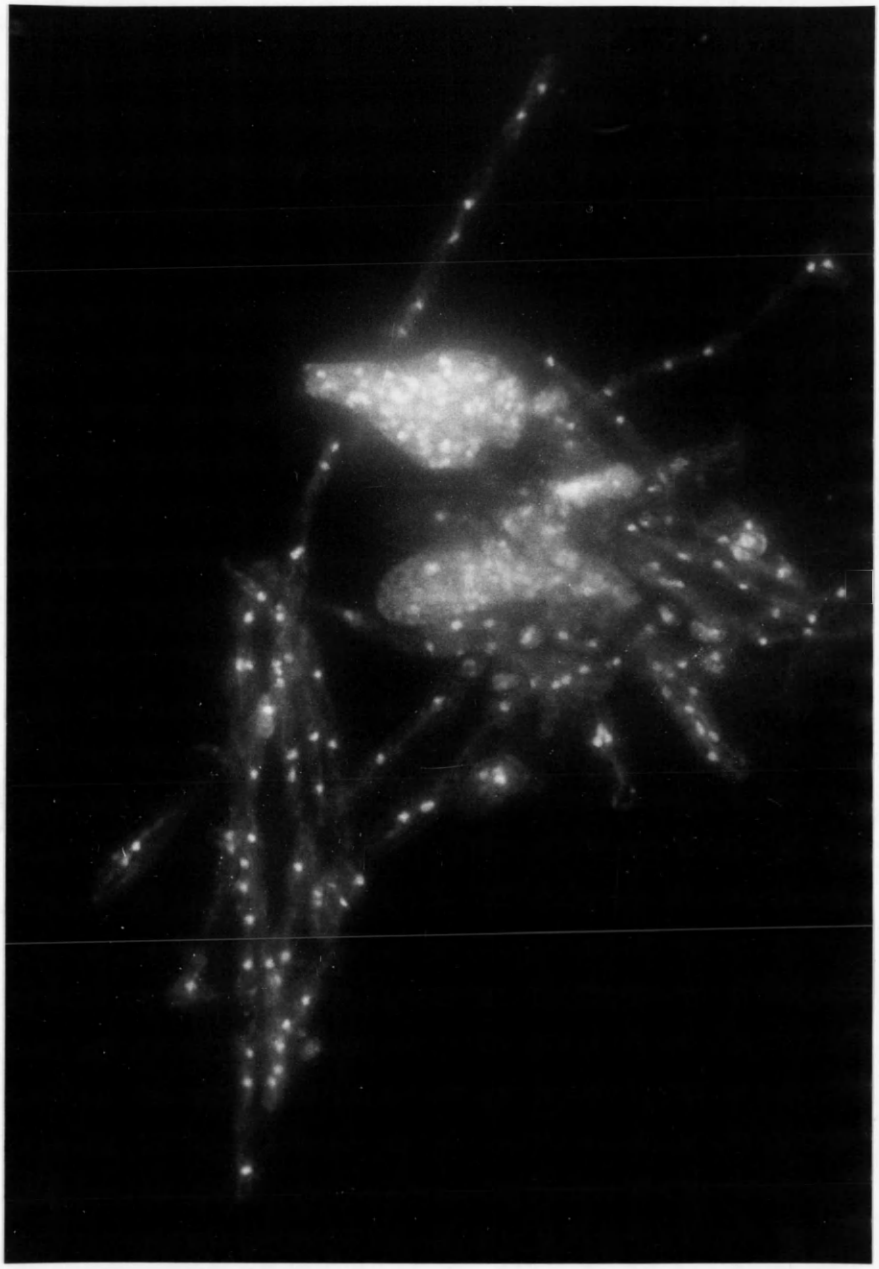


Plate 3a. Unstained 144 hour SR culture showing the presence of autofluorescent polyphosphate bodies. 40X magnification.

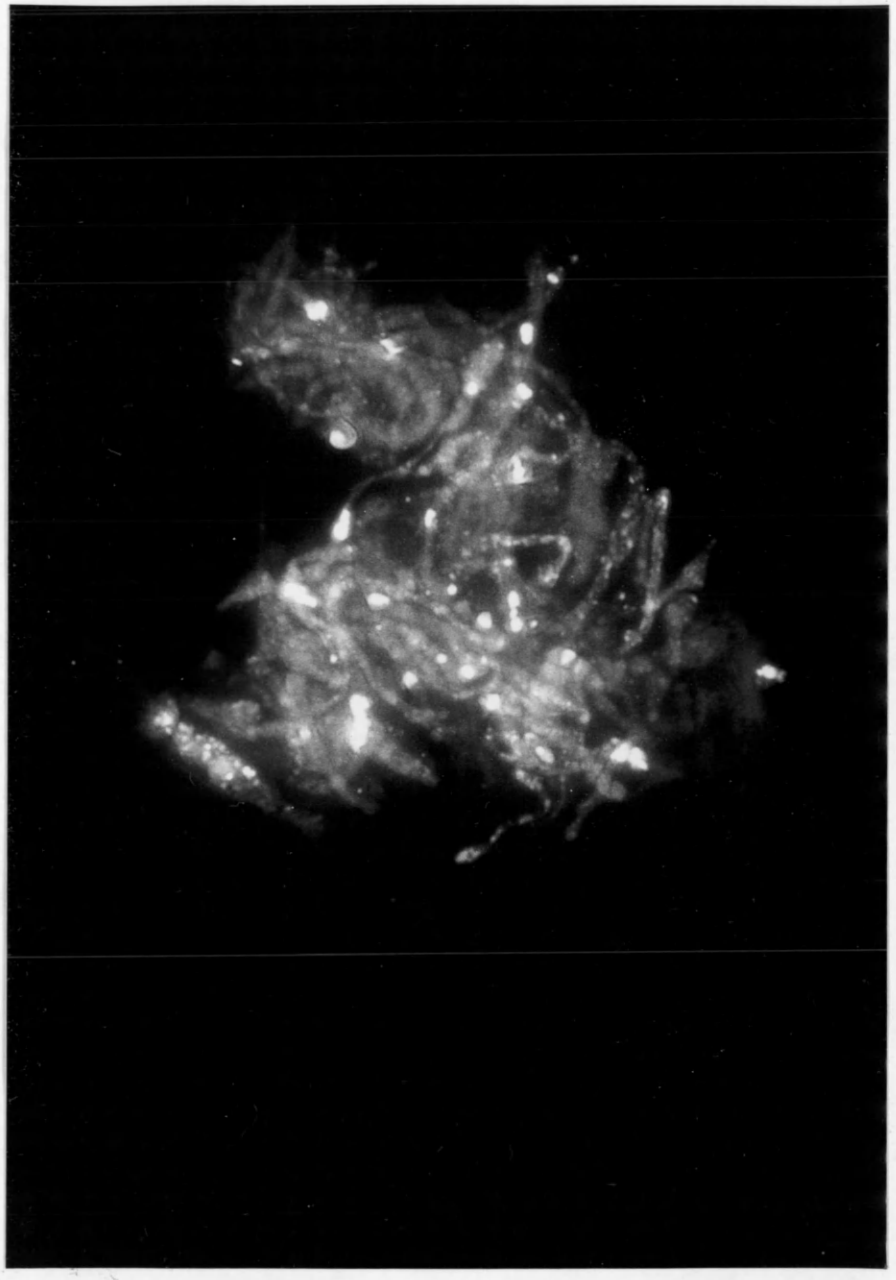


Plate 3b. Unstained 336 hour SR culture showing the presence of autofluorescent polyphosphate bodies. 20X magnification.

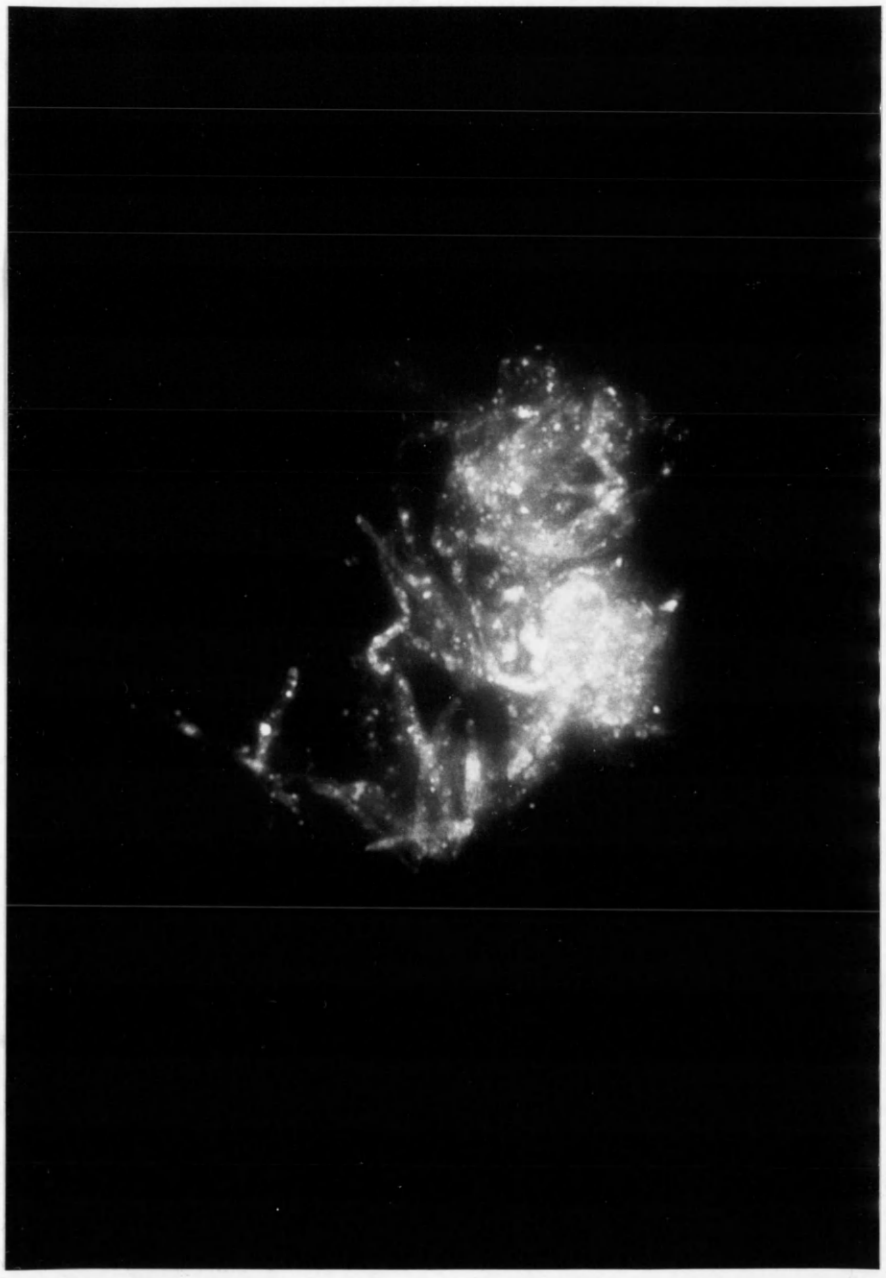


Plate 4a. Unstained 144 hour C culture showing the lack of autofluorescent polyphosphate bodies. 40X magnification.

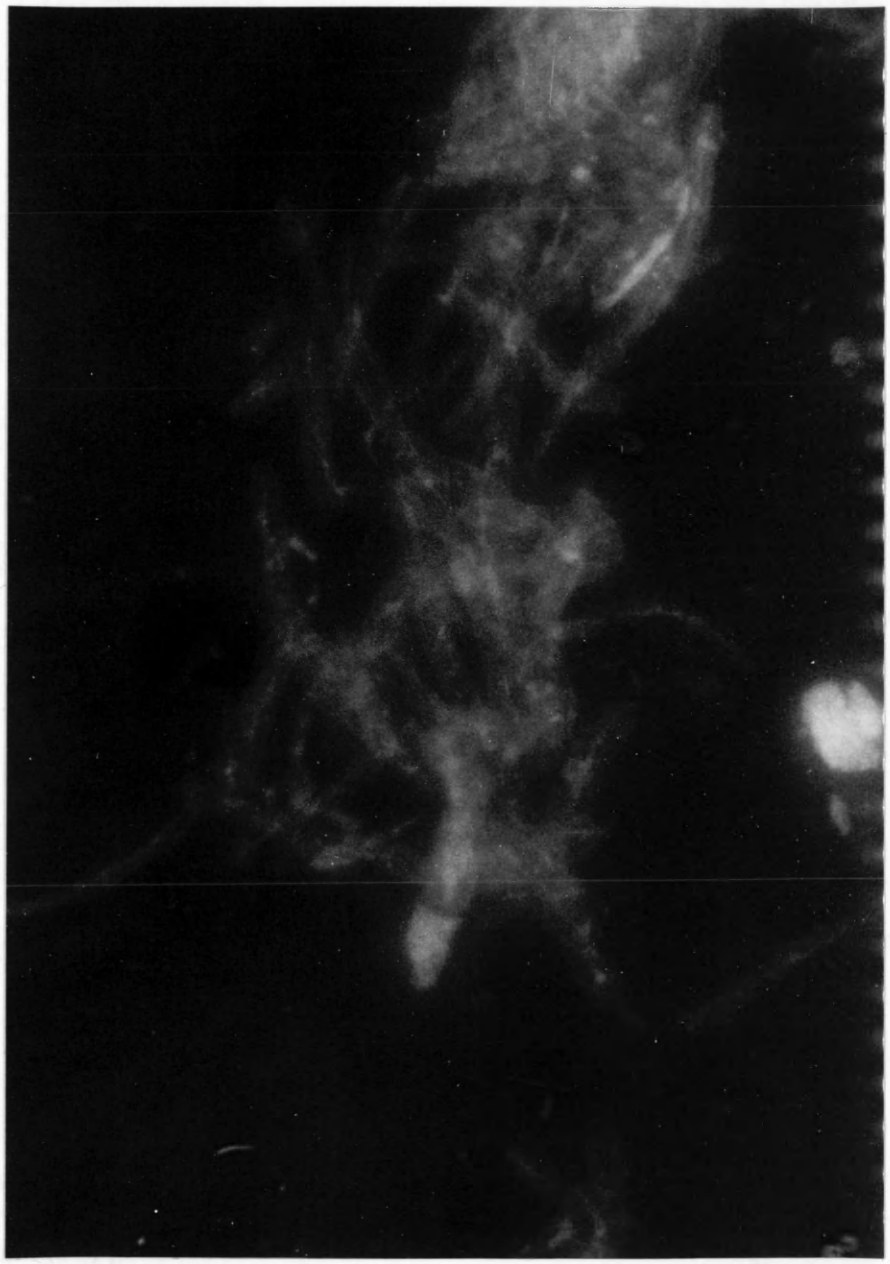


Plate 4b. Unstained 336 hour C culture showing the lack of autofluorescent polyphosphate bodies. 40X magnification.

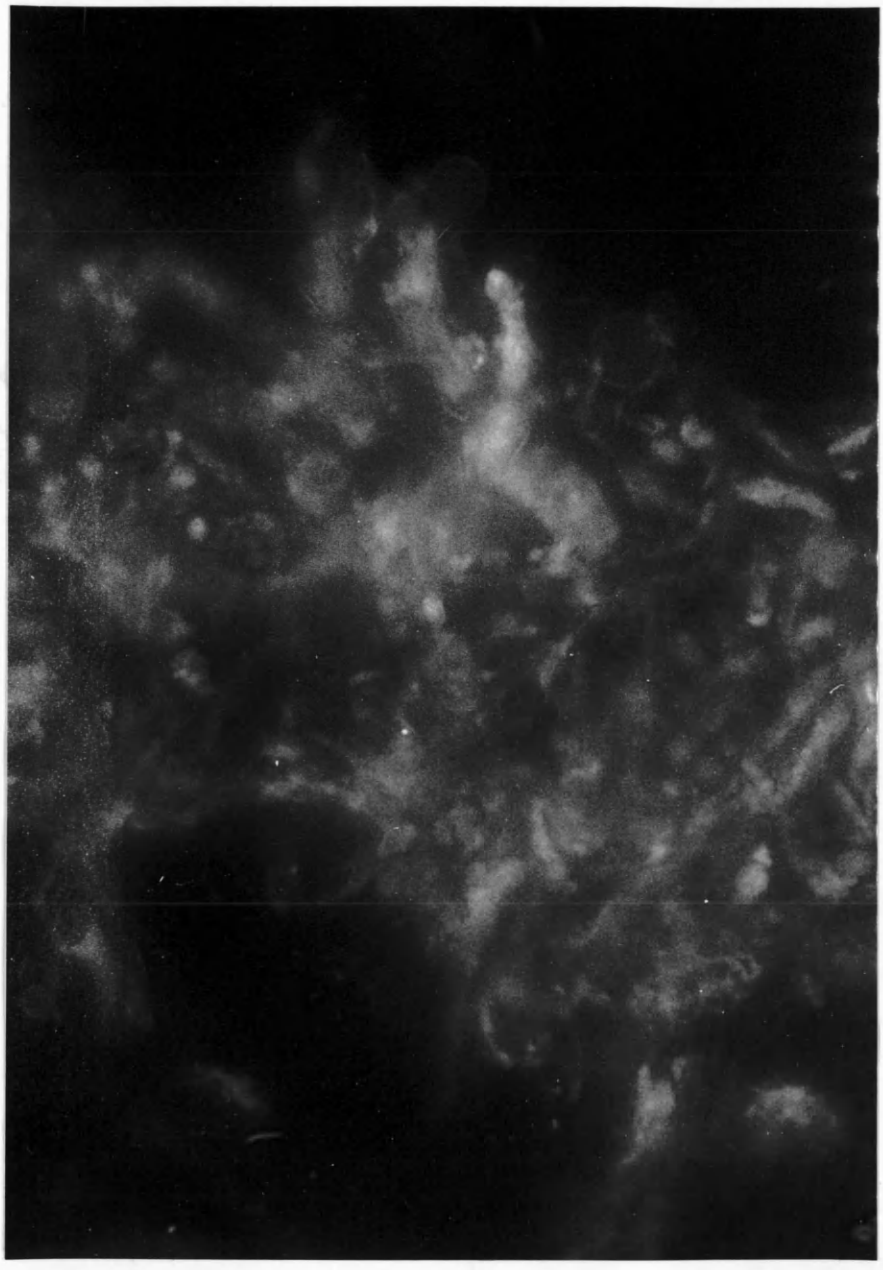


Figure 8. OPCA carboxylyase specific activity in nmol/min/mg of protein in different diet groups of Aspergillus ornatus at 144 hours of growth. For group descriptions, see text. Open bars denote induced activity levels. Lined bars denote basal activity levels. Each open bar represents the mean of six replicates from two separate experiments. Each lined bar represents the mean of four replicates from two separate experiments. The vertical lines are the standard error of the mean.

144 HOUR OPCA CARBOXYLYASE ACTIVITY

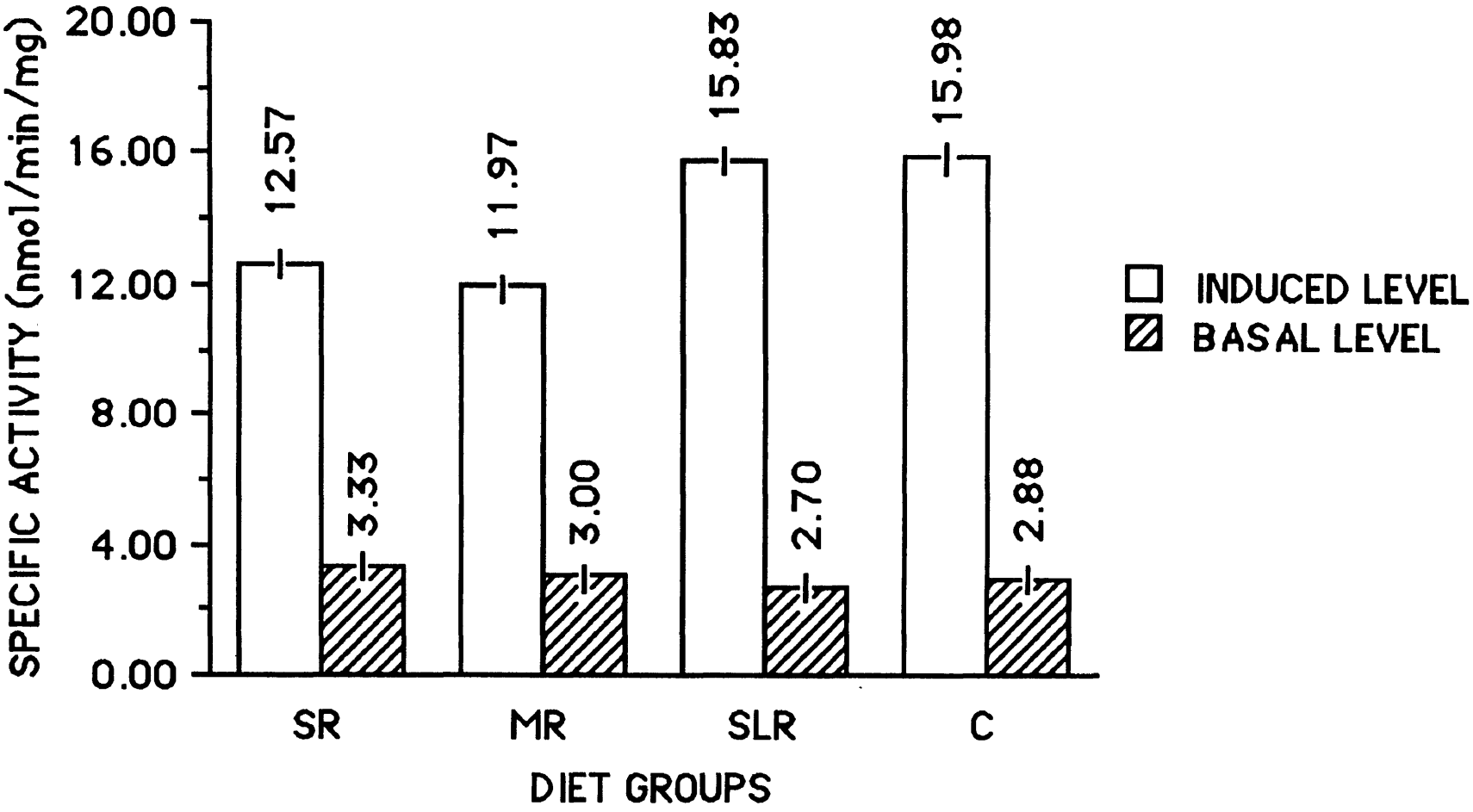


Figure 9. OPCA carboxylyase specific activity in nmol/min/mg of protein in different diet groups of Aspergillus ornatus at 336 hours of growth. For group descriptions, see text. Open bars denote induced activity levels. Lined bars denote basal activity levels. Each open bar represents the mean of six replicates from two separate experiments. Each lined bar represents the mean of four replicates from two separate experiments. The vertical lines are the standard error of the mean.

336 HOUR OPCA CARBOXYLYASE ACTIVITY

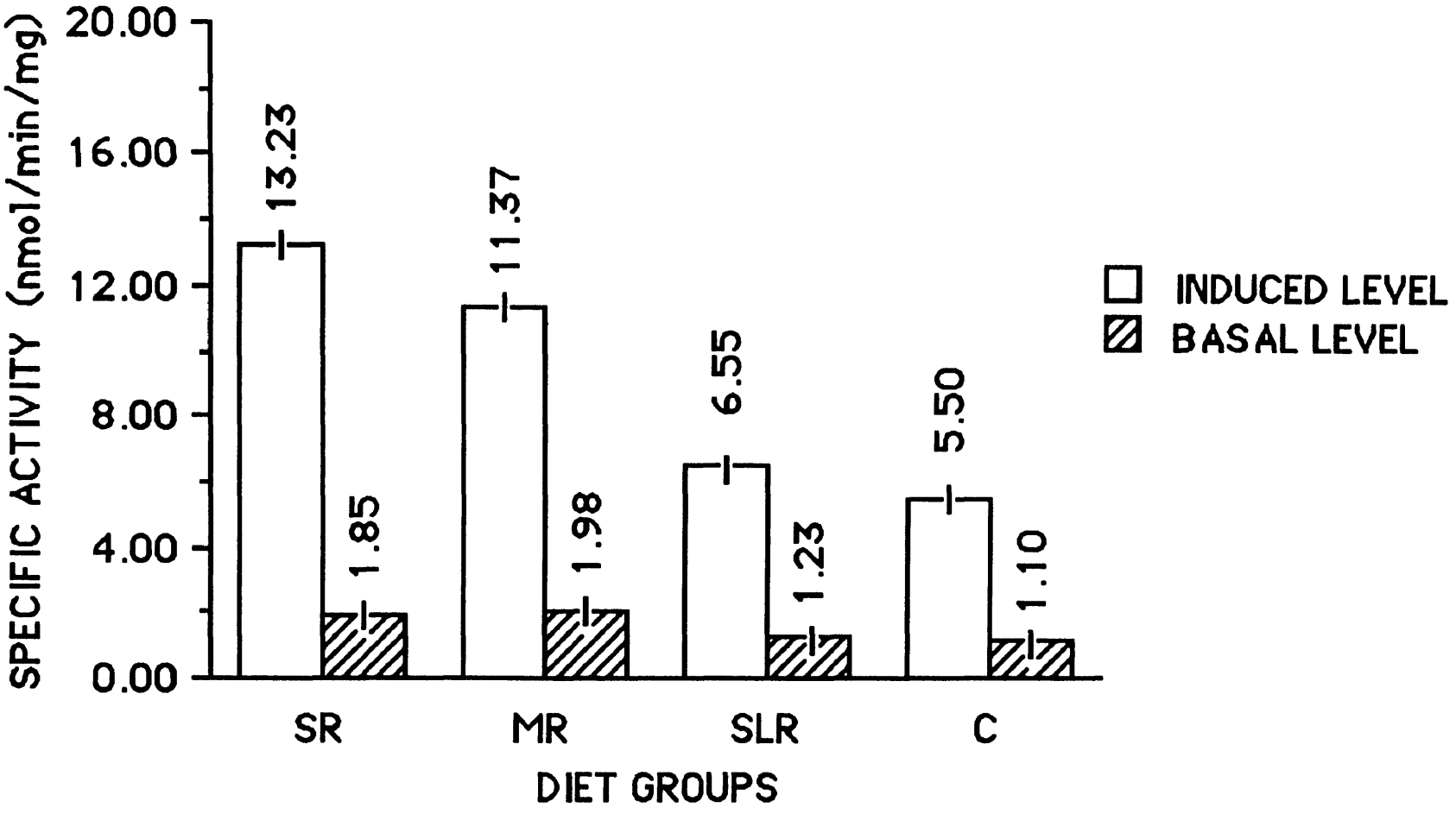


Table 3. OPCA carboxylyase specific activity in nmol/min/mg in different diet groups of Aspergillus ornatus vs age (hours of growth). For group descriptions, see text. The letter "I" or "B" after the age denotes whether the assay was performed on induced or basal cultures, respectively. Values are mean ±SEM for n=6 induced samples and n=4 basal samples in each diet/age group. Statistical significance of differences between group means were evaluated by the Student-Newman-Keuls multiple range test, which was applied when oneway analysis of variance indicated significant differences. Means in each row not sharing a common superscript letter were significantly different (p=0.05). Means in each column not sharing a common superscript number were significantly different (p=0.05).

TABLE 3

OPCA Carboxylyase Activity vs Age

Age (hours)	F Value	F Prob.	Diet Groups SR	MR	SLR	C
Induced Cultures						
144-I	29.2	p<.001	12.57 ±0.30^{a1}	11.97 ±0.29^{a1}	15.83 ±0.43^{b1}	15.98 ±0.50^{b1}
336-I	159.9	p<.001	13.23 ±0.43^{a1}	11.37 ±0.30^{b1}	6.55 ±0.25^{c2}	5.50 ±0.10^{d2}
432-I	N/A	N/A	3.87 ±0.29 ^{2}	N/A	N/A	N/A
Basal Cultures						
144-B	1.0	p>.40	3.33 ±0.17^{a3}	3.00 ±0.20^{a2}	2.70 ±0.27^{a3}	2.88 ±0.36^{a3}
336-B	21.9	p<.001	1.85 ±0.20^{a4}	1.98 ±0.17^{a3}	1.23 ±0.19^{b4}	1.10 ±0.15^{b4}
432-B	N/A	N/A	1.45 ±0.15 ^{4}	N/A	N/A	N/A

Figure 10. OPCA carboxylyase specific activity in nmol/min/mg of protein in different diet groups of Aspergillus ornatus at 336 hours of growth vs 336 hour cultures placed on a recovery medium (see text for description) for six hours prior to enzyme induction. Open bars denote 336 hour activity levels. Lined bars denote post-recovery activity levels. Each bar represents the mean of six replicates from two separate experiments. The vertical lines are the standard error of the mean.

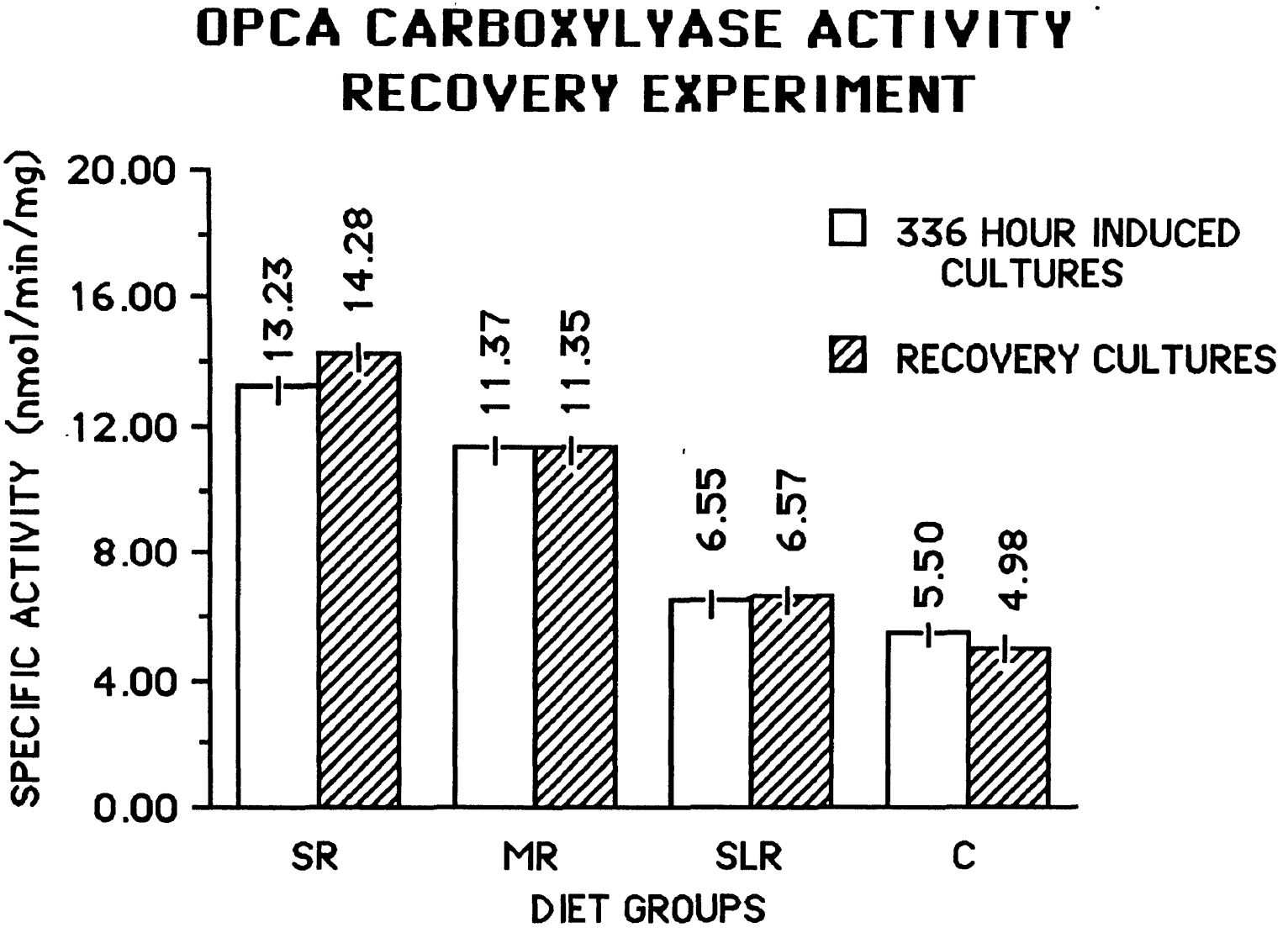

OPCA CARBOXYLYASE ACTIVITY
RECOVERY EXPERIMENT
SPECIFIC ACTIVITY (nmol/min/mg)
20.00
16.00
12.00
8.00
4.00
0.00
13.23
14.28
11.37
11.35
6.55
6.57
5.50
4.98
336 HOUR INDUCED CULTURES
RECOVERY CULTURES
SR
MR
SLR
C
DIET GROUPS

Figure 11. Comparison of OPCA carboxylyase specific activity in nmol/min/mg of protein between induced SR and C groups with time. For group descriptions, see text. Open bars denote enzyme activity of SR cultures. Lined bars denote enzyme activity of C cultures. Each bar represents the mean of six replicates from two separate experiments. Vertical lines are the standard error of the mean.

OPCA ACTIVITY IN SR AND C GROUPS WITH AGE

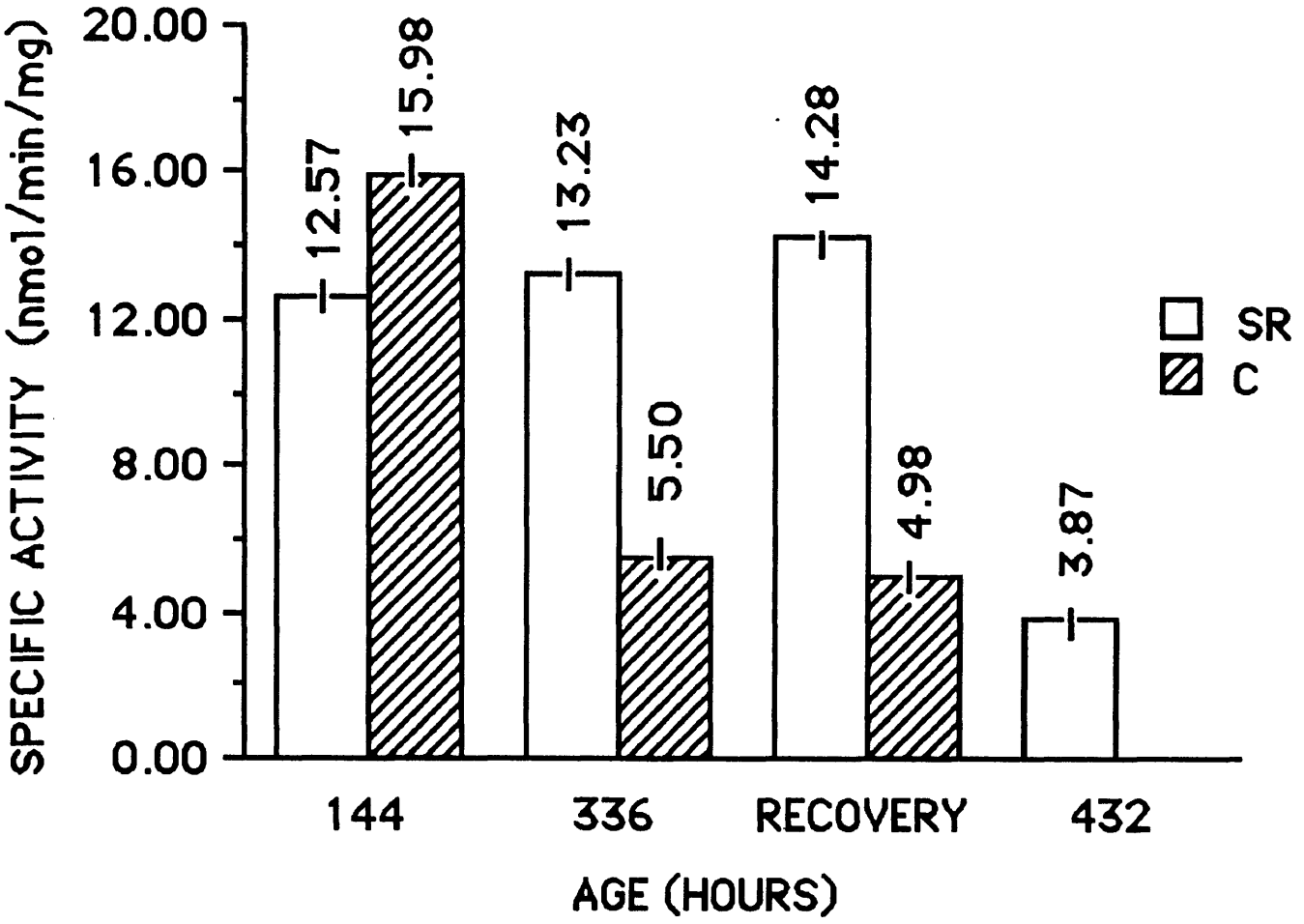


Table 4. Total protein in mg in the crude homogenate of different diet groups of Aspergillus ornatus vs age (hours of growth). For group descriptions, see text. The letter "I" or "B" after the age denotes whether the assay was performed on induced or basal cultures, respectively. Values are mean ±SEM for n=6 induced samples and n=4 basal samples in each diet/age group. Statistical significance of differences between group means was evaluated by the Student-Newman-Keuls multiple range test, which was applied when a oneway analysis of variance indicated significant differences. Means in each row not sharing a common superscript letter were significantly different (p=0.05). Means in each column not sharing a common superscript number were significantly different (p=0.05).

TABLE 4

Total Protein vs Age

Age (hours)	F Value	F Prob.	Diet Group SR	MR	SLR	C
Induced Cultures						
144-I	96.4	p<.001	3.85 ±0.04^{a1}	3.53 ±0.08^{b1}	2.62 ±0.07^{c1}	2.55 ±0.06^{c1}
336-I	435.3	p<.001	2.63 ±0.04^{a2}	3.18 ±0.04^{b2}	5.57 ±0.14^{c2}	6.55 ±0.10^{d2}
432-I	N/A	N/A	2.07 ±0.08 ^{3}	N/A	N/A	N/A
Basal Cultures						
144-B	25.0	p<.001	1.78 ±0.09^{a4}	1.70 ±0.12^{a3}	2.55 ±0.06^{b1}	2.40 ±0.10^{b1}
336-B	465.2	p<.001	1.55 ±0.06^{a5}	1.93 ±0.17^{b3}	5.80 ±0.13^{c2}	6.65 ±0.10^{d2}
432-B	N/A	N/A	1.37 ±0.13 ^{5}	N/A	N/A	N/A

Figure 12. Total protein in mg present in the crude homogenate of basal cultures of the different diet groups of Aspergillus ornatus vs age (hours of growth). For group descriptions, see text. Open bars denote 144 hour cultures. Lined bars denote 336 hour cultures. Each bar represents the mean of six replicates from two separate experiments. Vertical lines are the standard error of the mean.

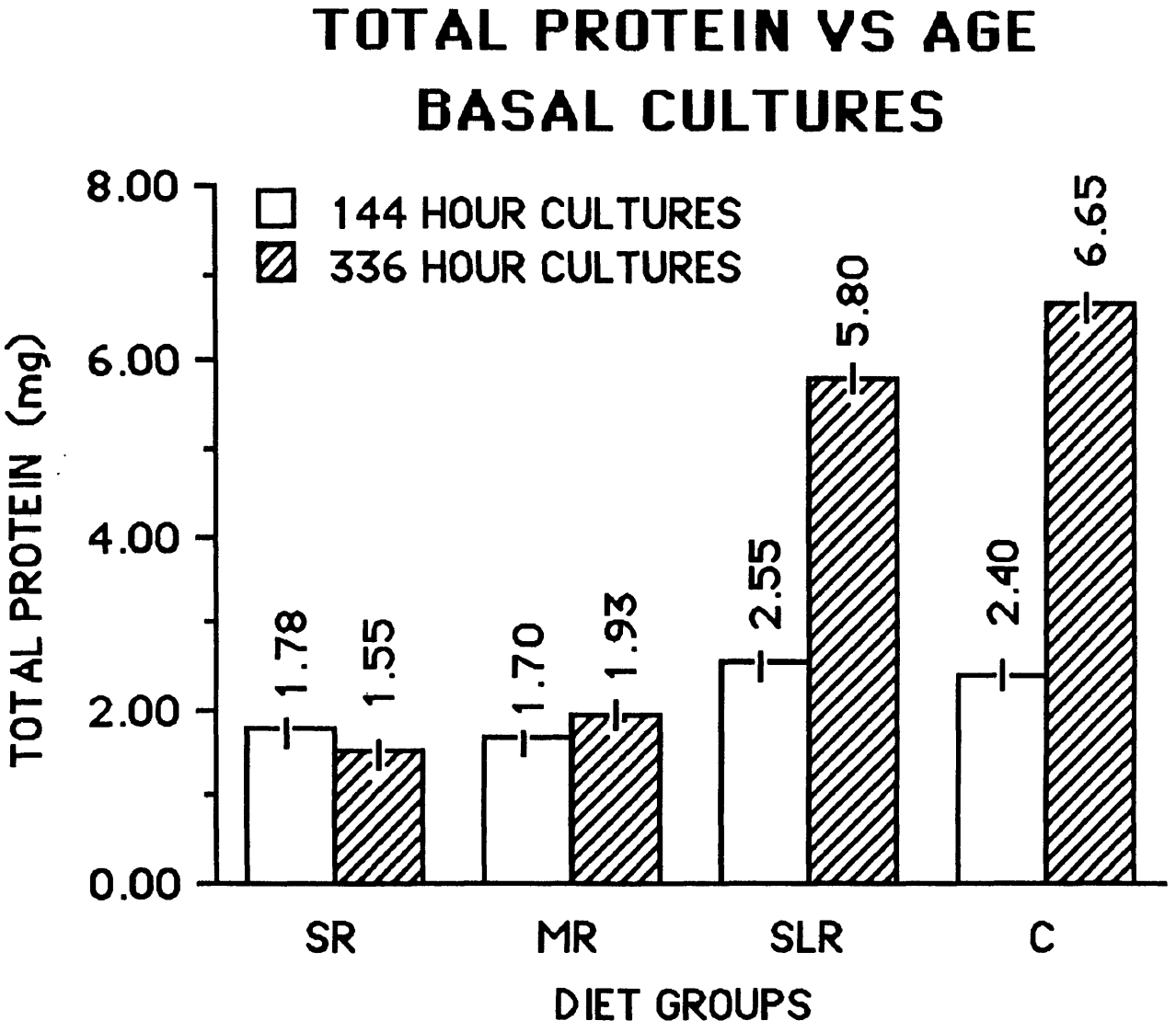

TOTAL PROTEIN VS AGE
BASAL CULTURES
144 HOUR CULTURES
336 HOUR CULTURES
TOTAL PROTEIN (mg)
8.00
6.00
4.00
2.00
0.00
1.78
1.55
1.70
1.93
2.55
5.80
2.40
6.65
SR
MR
SLR
C
DIET GROUPS

Figure 13. Total protein in mg present in the crude homogenate of induced cultures of the different diet groups of Aspergillus ornatus vs age (hours of growth). For group descriptions, see text. Open bars denote 144 hour cultures. Lined bars denote 336 hour cultures. Each bar represents the mean of six replicates from two separate experiments. Vertical lines are the standard error of the mean.

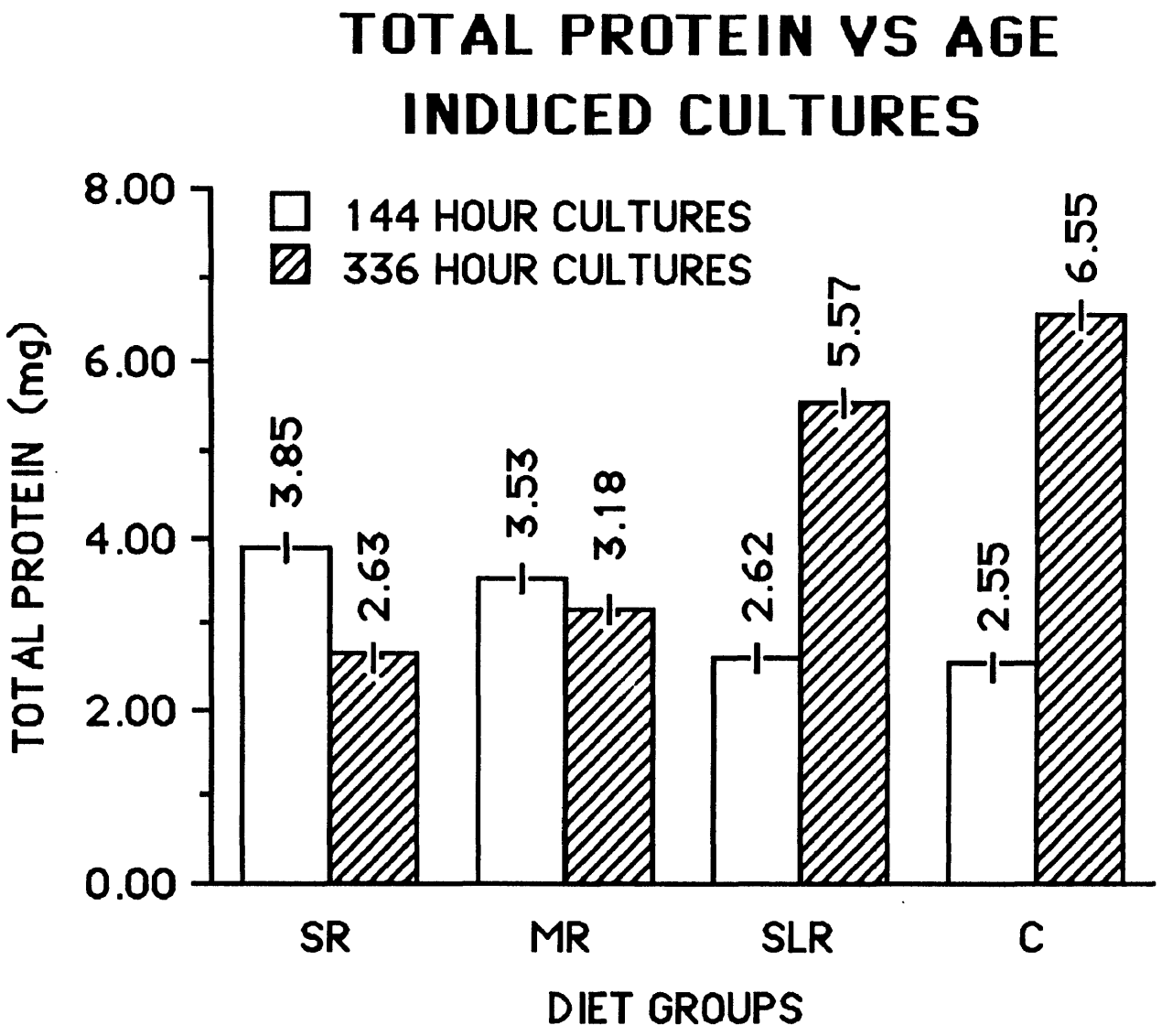

TOTAL PROTEIN VS AGE
INDUCED CULTURES
144 HOUR CULTURES
336 HOUR CULTURES
TOTAL PROTEIN (mg)
8.00
6.00
4.00
2.00
0.00
3.85
2.63
3.53
3.18
2.62
5.57
2.55
6.55
SR
MR
SLR
C
DIET GROUPS

Figure 14. Total protein in mg of SR cultures vs age (hours of growth). For group descriptions, see text. Open bars denote protein levels in induced cultures. Lined bars denote protein levels in basal cultures. Each open bar represents the mean of six replicates from two separate experiments. Each lined bar represents the mean of four replicates from two separate experiments. Vertical lines are the standard error of the mean. Results of the recovery experiment (see text for description) are illustrated under the heading "RECOVERY".

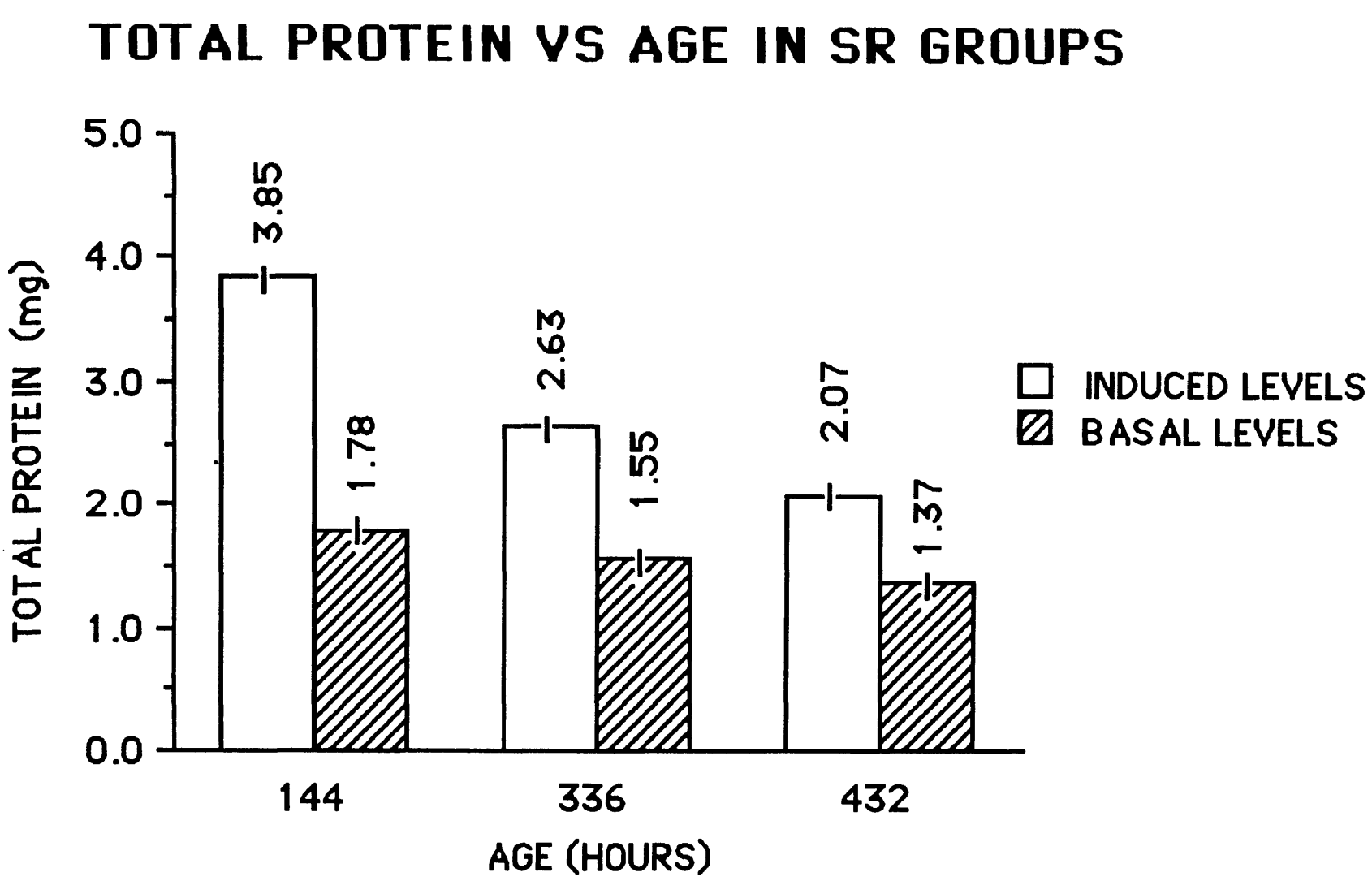
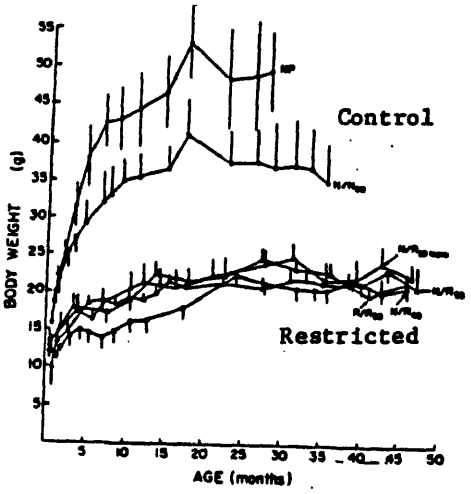

TOTAL PROTEIN VS AGE IN SR GROUPS
TOTAL PROTEIN (mg)
5.0
4.0
3.0
2.0
1.0
0.0
3.85
1.78
2.63
1.55
2.07
1.37
144
336
432
AGE (HOURS)
INDUCED LEVELS
BASAL LEVELS

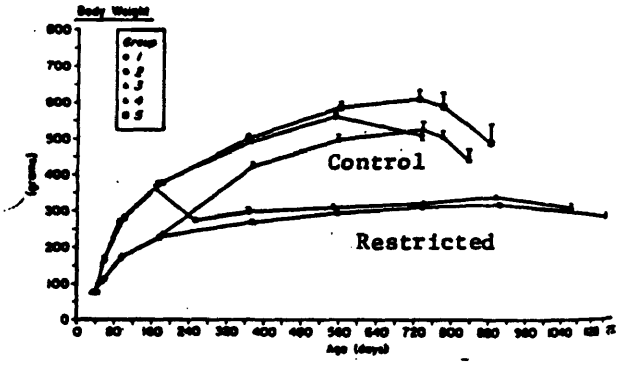
Figure 15. Dry weight comparison between mouse (Weindruch, et al., 1986), rat (Yu, et al., 1985) and Aspergillus ornatus with increasing age.

MOUSE STUDIES



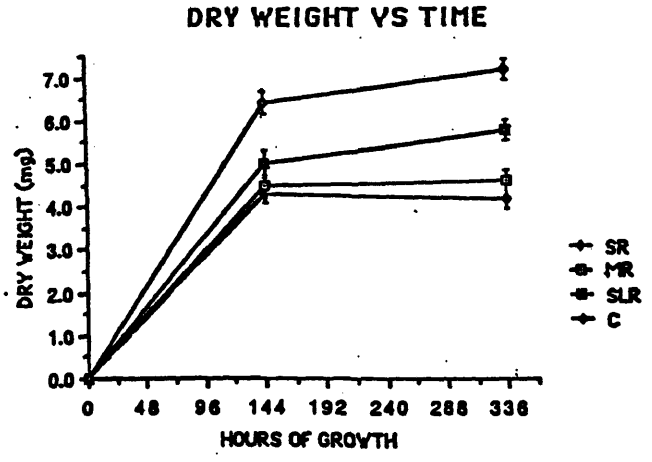
From: Weindruch, et al., 1986

RAT STUDIES



From: Yu, et al., 1985

ASPERGILLUS ORNATUS



Shah and Coursen, 1989

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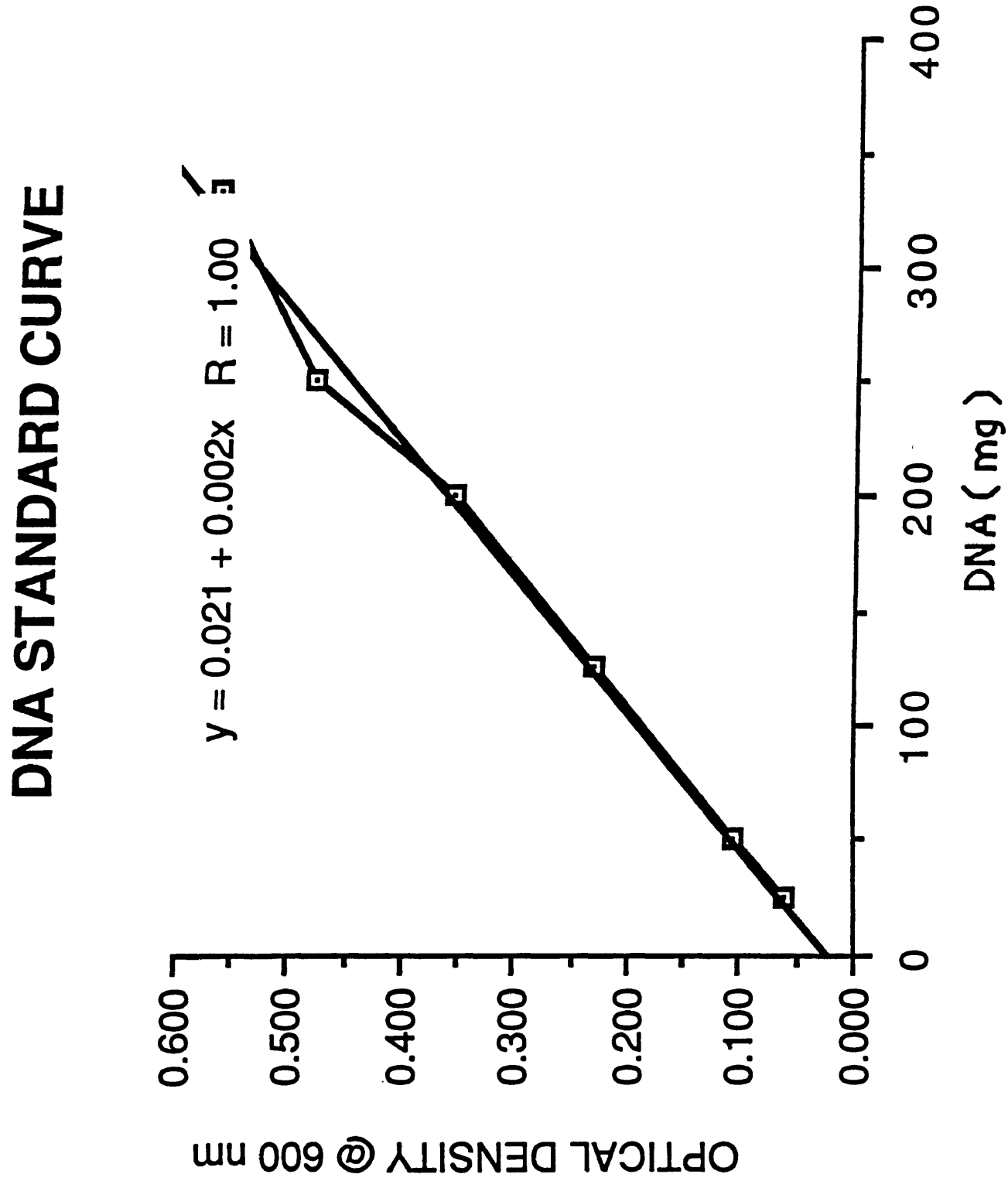
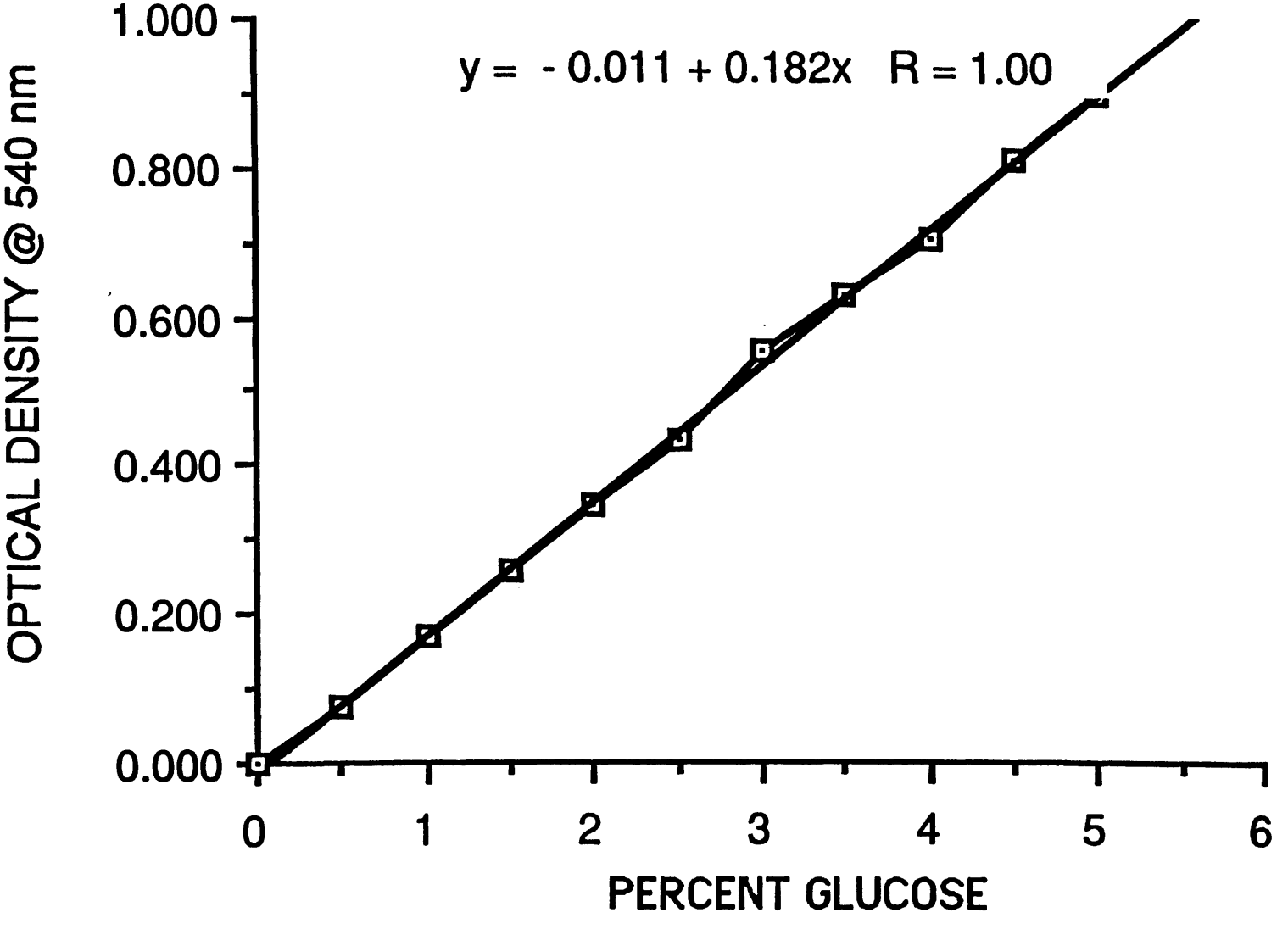
APPENDIX

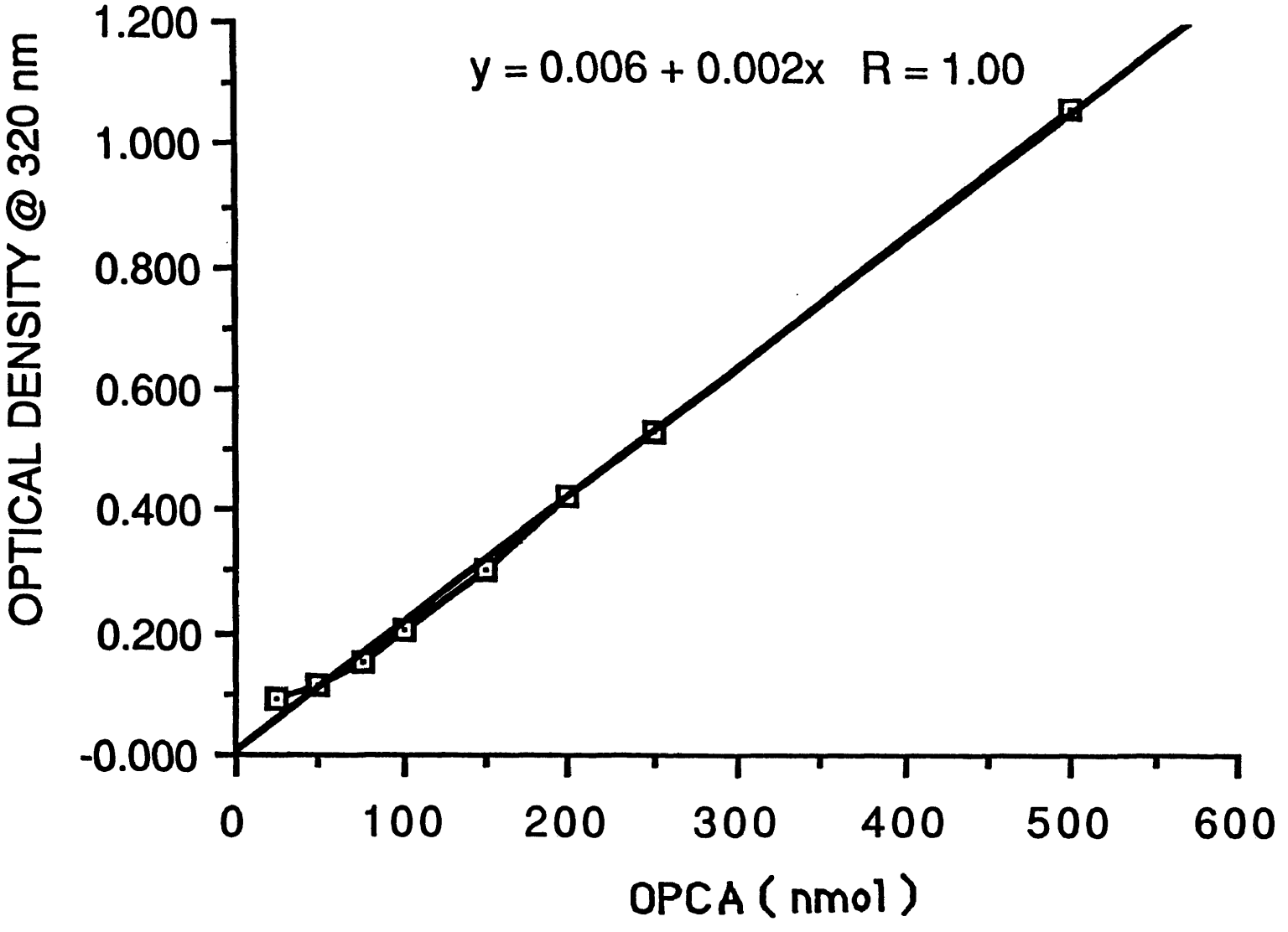
STANDARD CURVES FOR ASSAYS


DNA STANDARD CURVE
y = 0.021 + 0.002x R = 1.00
OPTICAL DENSITY @ 600 nm
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
100
200
300
400
DNA (mg)

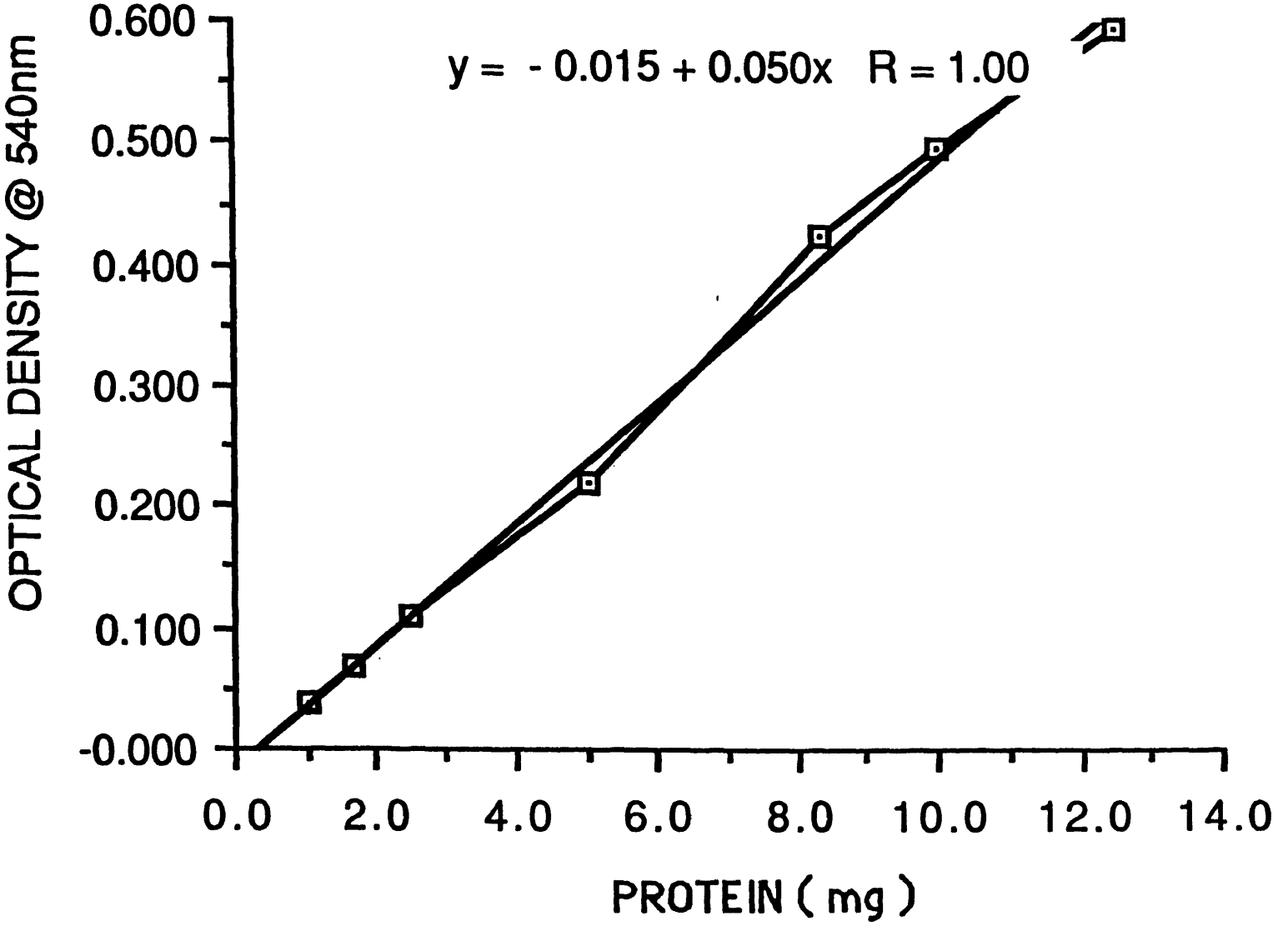
GLUCOSE STANDARD CURVE



OPCA STANDARD CURVE



PROTEIN STANDARD CURVE



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"I myself have not learned big things in my own research. I'm not a Watson or a Crick or a Weinberg, for that matter. I've learned small things. But to learn something one day that nobody ever knew before is something that, I think, everyone should have a chance to do."

—Carnegie Institution president Maxine Singer on the 2 November "Bill Moyers' World of Ideas" on PBS.